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14. ABSTRACT

There is a surprising disparity between the number of protein-encoding genes (ca. 30,000) in the human genome and the number of proteins (ca. 300,000) in the human proteome has inspired the development of translational proteomics aimed at protein expression profiling of disease states. Translational proteomics, which offers the promise of early disease detection and individualized therapy, requires new methods for analysis of clinical specimens. We have developed Quantitative Fluorescence Imaging Analysis (QFIA) for accurate, reproducible quantification of proteins in slide-mounted tissues. The method has been validated for analysis of β-catenin in archived prostate specimens fixed in formalin.

β-catenin expression was analyzed in a cross-sectional case-control study that included 42 cancer cases and 42 controls matched on the basis of age (±5 years) and year of biopsy (±3 years). Reduced expression of β-catenin in Normal Appearing Acini (NAA) relative to the Normal Acini (NA) of matched controls is a potential field marker for Prostate Cancer, in biopsies that miss existing adenocarcinomas. The observed sensitivity (42%) and specificity (88%) qualify the marker as a potentially significant contributor to a small panel of field markers, and support the feasibility of applying QFIA to the development of such a panel.

15. SUBJECT TERMS
risk assessment, prostate cancer, biomarkers

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INTRODUCTION

Diagnosis of prostate cancer (PC), the second leading cause of cancer deaths among U.S. men [1], is dependent on quantification of serum PSA. A PSA test of 4-10 ng/mL, however, has a specificity of only 25% [2] and requires biopsy, which has a false negative rate of 20% [3], necessitating rebiopsy of the large majority of these men. A potential solution to this problem is based on the concept of biomarker alterations associated with premalignant field disease and cancer-induced field effect [4-6]. Premalignant field disease is defined as genetic and/or epigenetic modifications of morphologically normal-appearing cells that herald an increased probability of neoplastic transformation [6,7]. In response to continued exposure to carcinogenic insult, cells at multiple foci are initiated (polyclonal disease) and undergo progression toward PC [8]. In contrast, field effect is defined as the presence of epigenetic changes in cells outside of the tumor nodules, in response to altered signaling from cancer cells [6,9]. Cells within the premalignant field or the area of field effect exhibit a normal morphology but express phenotypic modifications that may be detected as qualitative or quantitative biochemical changes [6,10]. Consequently, it may be possible to identify patients with early cancer on the basis of a positive cancer biopsy or a morphologically normal-appearing biopsy expressing biochemical field disease/effect.

An objective of the present study was to evaluate the adhesion/signaling protein β-catenin as a potential field marker for prostate cancer. β-Catenin is a 92-kD protein encoded by a gene localized to human chromosome 3p22 [11]. The protein expresses both cell adhesion and transcriptional regulation functions that are independent of each other. Impaired β-catenin degradation with increased cellular concentration of the protein, contributes to inappropriate activation of transcription factors and uncontrolled cell proliferation. Transmembrane cadherin molecules, the prime mediators of calcium-dependent cell-cell adhesion, express an intracellular catenin-binding domain [12]. Binding of β- or γ-catenin to this domain is required for linkage of E-cadherin to actin filaments of the cytoskeleton. Deletion of the catenin-binding domain of E-cadherin or functional inactivation of catenins disables cell-cell adhesion even though E-cadherin retains its extracellular binding domain. In the prostate, β-catenin appears to act primarily as a cell adhesion molecule rather than a transcription factor [13]. The cell-cell adhesion complex is compromised in cancer-bearing prostate glands, as evidenced by marked down regulation of cadherins in the cancerous epithelium [14].

Reverse-phase protein array analysis (RPPA) [15-19] and automated quantitative analysis (AQUA) [20-22] are two recently introduced methodologies that precisely quantify proteins and their secondary modifications in clinical specimens, *i.e.*, slide-mounted tissues. RPPA was developed with a focus on modifications of cell-signaling cascades in disease, and simultaneously analyzes multiple lysates from one or more patients.

Work presented in this report addressed two, integrated objectives; 1) validation of a new method, tissue-based QFIA, for fluorescence quantification of β-catenin in slide specimens of archived prostate tissues that were fixed in formaldehyde-based fixative, and 2) evaluation of β-catenin expression as a potential field marker for prostate cancer, using archived prostate tissue specimens.

ACQUISITION OF TISSUE SPECIMENS

The laboratory has accrued 11 new cases of prostate cancer and BPH, during the past year (Sep 1, 2004 – Aug 31, 2005). Tissue sections, cores, and fine needle aspirates (FNAs) were harvested systematically, from the peripheral zone (with some associated central zone) of each resected gland and fixed as described previously, embedded in paraffin or cryopreserved, archived, and logged into our database.

The surgical pathology database of the Veterans Affairs Hospital at Omaha (VAHO) was searched for archived cancerous core biopsies and non-cancerous biopsies of patients who were followed for at least 5-years and remained without clinical prostate cancer. Patients without clinical cancer (controls) were matched one-to-one with cancer patients on the basis of age (± 5 years) and year of biopsy (± 3 years). (See attached manuscript for more details)

FIXATION OF TISSUE SPECIMENS

The optimal fixation method yielding the brightest fluorescence signal was used as outlined in last year's report. Briefly, it is fixing prostate tissue fragments and cores in 5% formaldehyde, 4% paraformaldehyde (for cryosectioning), and Streck Fixative. Cell lines are routinely fixed in QFIA fixative and stored at -80^{o}C, prior to imprinting to glass slides.

DEPARAFFINIZATION AND EPITOPE RECOVERY

Slight modifications were introduced to the deparaffinization and epitope recovery steps to achieve best results. Slide specimens of paraffin-embedded cores and tissue sections were deparaffinized with EZ-AR Common™ solution (BioGenex Laboratories) and treated for epitope recovery with EZ-AR1™ according to manufacturer specifications. Specimens were processed with a computer-controlled, microwave system (EZ-Retriever; BioGenex Laboratories). Slides were rinsed with tap water and then partially dried for application of labeling barriers with a PAP pen.

QUANTITATIVE FLUORESCENCE LABELING

Few modifications were introduced to the quantitative fluorescence labeling procedure in order to enhance labeling and to achieve reproducible results.

Labeling was completed with the BioGenex autostainer optimized to obviate slide batching, which was associated with a 25% to 30% loss of β-catenin signal. Partially dried slides were loaded into the autostainer programmed to soak slides for 15 min with OptiMax buffer, block for 20 min with 10% normal goat serum, label for one hour with mouse monoclonal antibody (1^{o} Ab) specific for β-catenin and then label for one hour with goat anti-mouse IgG antibody (2^{o} Ab) coupled with Alexa Fluor® 568. This two-antibody system superseded an avidin/biotin labeling system that produced higher backgrounds and lower signal-to-noise ratios. Quantitative fluorescence labeling requires saturation of epitopes by their corresponding Abs. Saturating dilutions of the 1^{o} Abs were determined by titration against β-catenin in LNCaP cells and slide preparations of a standard benign hyperplastic (BH) gland. The optimum dilution for saturation labeling with primary Ab was 1/100 for tissue sections and cell lines. The optimum dilution for labeling with the 2^{o} Ab was also 1/100. Negative controls were treated with a non-

immune mouse IgG isotype adjusted to an immunoglobulin concentration the same as that of the diluted 1^{o} Ab. The autostainer program ended, by rinsing the slides with OptiMax® buffer. All labeling procedures were completed at room temperature. Labeled specimens were mounted with ProLong® Gold antifade reagent (Molecular Probes, Inc.), sealed with clear lacquer, and stored at –20^{o} C. Other antifade reagents including N-propylgallate [23] and *SlowFade*® Antifade provided markedly less protection against photobleaching and the loss of fluorescence signal during storage of the slide specimens.

FLUORESCENCE IMAGING AND IMAGE CAPTURE

Quantifiable, images of fluorescent, slide specimens were generated with a fully automated Leica DMXRA2 microscope equipped with a Marzhauser eight-slide scanning stage and a 150-watt mercury/xenon (Hg/Xe) excitation lamp that offers stable illumination, essential for fluorescence quantification, and a long lifetime of 2000 hours. Twelve-bit grayscale images were captured for quantification of fluorescence signal, with a digital b/w CCD camera. Twelve-bit digitized images were stored as 'tif' files. All components of the automated imaging analysis system were controlled and fully programmable with Image-Pro® Plus software

Each image capture session was initiated with a calibration protocol to confirm consistent, system performance across analytical runs **(Figure 1)**. Individual slides were prepared with each of three suspensions of InSpeck™ calibration microspheres (Molecular Probes) that produced relative emissions of 1%, 10%, and 100%. Fluorescence images were generated with the 20x objective and Chroma FITC filter set and captured as 12-bit gray scale files, at exposures of 250, 25, and 3 milliseconds, respectively, with the b/w CCD camera. The 12-bit gray scale consists of 4096 divisions or units (gsu). Images of 200-400 individual microspheres per slide were segmented at a threshold of 900 gsu and measurements including mean pixel intensity (MPI) of each microsphere were transferred to an Excel sheet for analysis. Mean and standard deviation of the MPIs for spheres from each suspension were computed and compared to the values of previous image capture sessions.

Positively labeled specimens of cases and controls, cell standards, or tissue standards were reviewed for fluorescence intensity; one specimen exhibiting the brightest fluorescence was selected from each group. Camera exposure time was set such that the brightest pixels in the selected specimen yielded values of *ca.* 3200 gsu, i.e., 80% of maximum on the 12-bit gray scale. All frames were captured at this exposure setting, typically 100-200 milliseconds at a gain of '0' for both single cells and tissue sections captured with the 10x objective. Camera frames were selected to maximize the number of events (single cells or acini) captured. In addition, frames of cancerous cores were selected to provide a balanced representation of cancerous acini/epithelium (CA) and normal-appearing acini (NAA). Categories of acini included CA, NAA, and normal acini (NA) of control cores and the tissue standard. Typically, 30-400 acini were captured in each category and 200-400 single cells were captured from each slide preparation of the LNCaP cells. Images were stored as 12-bit gray scale files.

QUANTIFICATION OF FLUORESCENCE SIGNAL IN CAPTURED IMAGES

Captured image files were loaded into the Image-Pro® Plus environment and relevant events, *i.e.*, individual LNCaP cells and the acini of cores and tissue sections were segmented for quantification of fluorescence signal and event dimensions, with the 'count/size' function of

Image-Pro® Plus. Precise segmentation of acini was achieved by setting a lower threshold just above background emission, typically *ca*. 230-250 gsu, and an upper threshold at the upper limit of the 12-bit gray scale, *i.e.*, 4095 gsu. In addition, an image filter set for a feret (event caliper measurement) of 10-15 microns excluded from measurement stromal elements, primarily endothelium, that labeled with the fluorescent reagents. Image-Pro® Plus generated a high-content array of measurements that included event number, area, feret, mean pixel intensity (MPI), sum of pixel intensities, and maximum and minimum pixel intensity. The algorithm for event MPI excluded the background pixels of each segmented event **(Figure 2)**. Fluorescence signal and spatial features of the LNCaP cells were quantified in the same way, except that a filter was set for an area of 3 μ^2 and excluded fluorescent fragments from measurement.

ANALYSIS OF BETA-CATENIN EXPRESSION IN BENIGN HYPERPLASTIC AND CANCER-BEARING PROSTATE GLANDS, BY QUANTITATIVE FLUORESCENCE IMAGING ANALYSIS (QFIA)

MPIs of labeled acini and single cells were corrected for non-specific immunoglobulin labeling and background emission by subtracting the average MPI (AMPI) of acini in the corresponding isotype control (typically 250-300 gsu). To determine whether the AMPI in each data set was representative, we plotted the AMPI of increasing numbers of events. AMPI was considered representative if the plot attained a steady 'zero' slope, typically beginning at 30 acini or 100 cells. Arithmetic average, standard deviation, and standard error were computed for each type of event (acini or single cells). Differences between the AMPIs of acinar categories (NA, NAA, CA) for each matched pair were evaluated by a paired T-test with PlotIT software.

Receiver operating characteristic (ROC) curves were generated for case-associated AMPIs of CA or NAA in relation to control-associated AMPIs of NA, plotted as a function of increasing threshold AMPI [24]. Fractional area-under-curve (AUC) along with 95% confidence intervals was determined for each plot. An AUC $\geq$ 0.67 indicated significant discrimination of cases (NAA or CA) and controls (NA). Sensitivities and specificities and their 95% confidence intervals were determined at selected threshold values.

KEY RESEARCH ACCOMPLISHMENTS

- Collected, processed and archived prostate specimens including FNAs, core biopsies, and tissue blocks from resected glands from 11 individuals.

- The surgical pathology database of the Veterans Affairs Hospital at Omaha (VAHO) was searched for archived cancerous core biopsies and non-cancerous biopsies of patients who were followed for at least 5-years and remained without clinical prostate cancer.

- Tissue fixation method was fine-tuned and optimized for best results.

- Deparaffinization and epitope recovery steps were modified, and computer-controlled, microwave system was used for better control of temperature and to enhance within-the-run and run-to-run reproducibility.

- Two-antibody system (primary Ab and Alexa Fluor® secondary Ab) was used for fluorescence labeling. This two-antibody system superseded an avidin/biotin labeling system that produced higher backgrounds and lower signal-to-noise ratios.

- A calibration protocol was developed and followed prior to every image capture session. This step is critical to confirm consistent, system performance across analytical runs **(Figure 1)**.

- Image-Pro® Plus software was programmed and used to generate a high-content array of measurements that included event number, area, feret, mean pixel intensity (MPI), sum of pixel intensities, and maximum and minimum pixel intensity **(Figure 2)**.

- Using LNCaP cell lines, we established within-the-run and run-to-run reproducibility of beta-catenin expression in cell lines **(Figure 3)**.

- Using benign hyperplasia tissue, we established within-the-run and run-to-run reproducibility of beta-catenin expression in tissues **(Figure 4)**.

- Saturating dilutions of the 1^{o} Abs were determined by titration against β-catenin in LNCaP cells and slide preparations of a standard BH gland. The optimum dilution for saturation labeling with primary Ab was 1/100 for tissue sections and cell lines. The optimum dilution for labeling with the 2^{o} Ab was also 1/100 **(Figure 5)**.

- ProLong® Gold antifade reagent was used and tested against other antifade reagents including N-propylgallate and *SlowFade*® Antifade. ProLong® Gold antifade provided markedly higher protection against photobleaching and the loss of fluorescence signal during storage of the slide specimens.

- A 150 watt mercury/xenon (Hg/Xe) excitation lamp was installed and used for signal excitation/emission. This lamp offers stable illumination, essential for fluorescence quantification, and a long lifetime of 2000 hours.

- Analysis of beta-catenin expression in benign hyperplastic and cancer-bearing prostate glands was accomplished using Quantitative Fluorescence Imaging Analysis (QFIA).

- Reverse-Phase Protein Array analysis (RPPA) analysis was used for the validation of QFIA ($r = 0.97$) **(Appendix)**.

- Established the foundation for application of tissue-based QFIA to the problem of quantifying β-catenin in archived prostate biopsies fixed in formalin, for an evaluation of protein expression in normal-appearing acini as a potential field marker for the presence of prostate cancer.

- Expression of β-catenin was analyzed in a cross-sectional case-control study that included 42 cancer cases and 42 controls matched on the basis of age (±5 years) and year of biopsy (±3 years).

- Showed that β-catenin expression in Normal Appearing Acini in a cancer-bearing gland (NAA) as determined by QFIA is potentially an effective marker for clinical diagnosis of prostate cancer in biopsies that may not include existing cancerous lesions, and support the application of QFIA to archived tissues for identification of potential markers of disease **(Appendix)**.

- A manuscript was accepted for publication July 2007, *Cancer Epidemiology Biomarkers & Prevention*). The manuscript includes more details on accomplishments and is attached in the Appendix

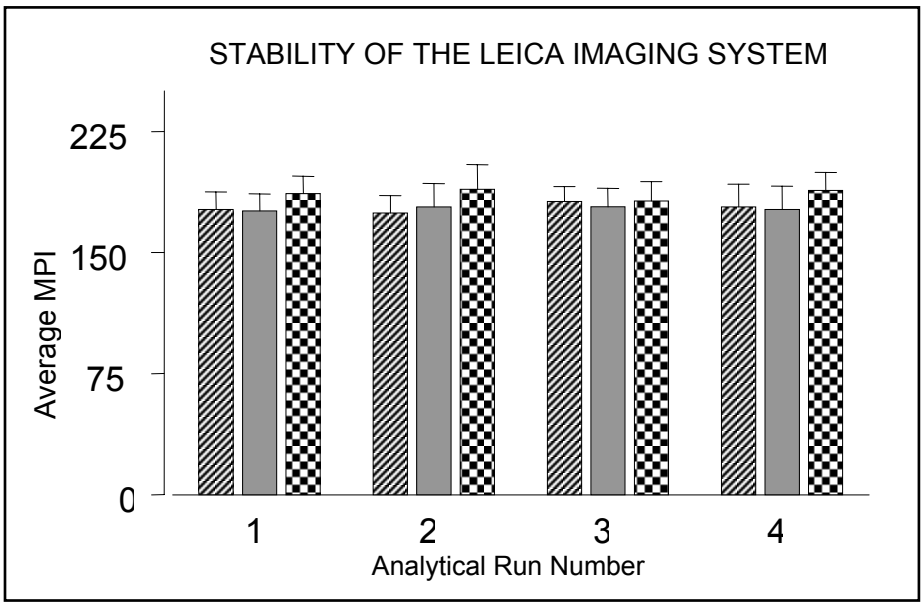

Figure 1. Fluorescence emission of standard microspheres (Molecular Probes) at 1% (cross hatched), 10% (gray), and 100 % (pattern) were captured with the Hamamatsu CCD b/w camera at exposures of 250, 25, and 3 milliseconds, respectively. Fluorescence emission was determined as Mean Pixel Intensity per microsphere (MPI). Each bar represents the average MPI ± standard deviation for 200-400 microspheres. Average MPI for each class of microsphere varied less than 10 % across analytical runs. Variations of signal exceeding 10 % indicate the need to replace the Hg/Xe lamp. In our experience the lamp remains stable for 1500-2000 hours of use, corresponding to hundreds of analytical sessions.

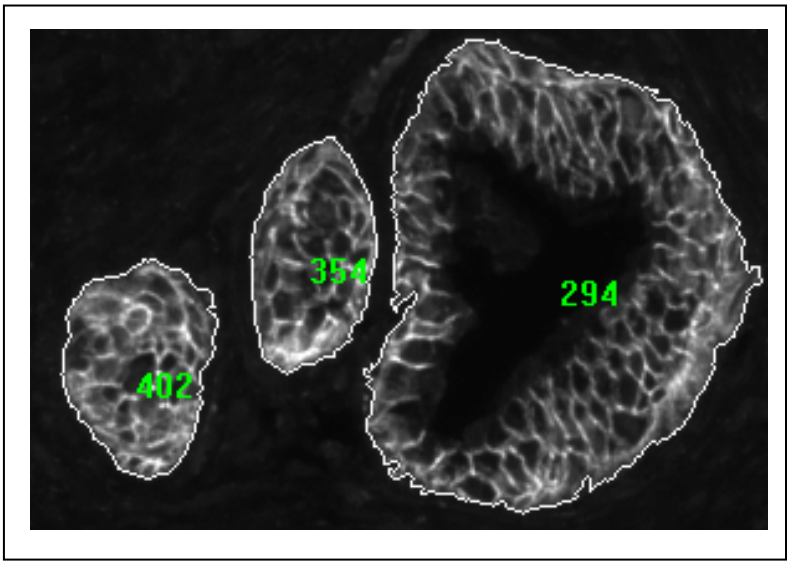

Figure 2. Archived, paraffin-embedded specimens from a benign hyperplastic prostate gland were sectioned at 4 microns and mounted to glass slides. Slide specimens were treated for epitope recovery and incubated with a mouse monoclonal anti-β-catenin antibody (Zymed Laboratories). Subsequently, slides were washed and incubated with a goat anti-mouse IgG antibody conjugated with Alexa Fluor 488. Images were captured with the Hamamatsu CCD camera and peripheral zone acini were automatically segmented, at a gray scale threshold of 240 gray scale units, with ImagePro Plus software. The software algorithm for computing Mean Pixel Intensity excludes pixels below threshold. B-Catenin labeling produces very bright, sharp signals enabling precise segmentation of the prostatic acini.

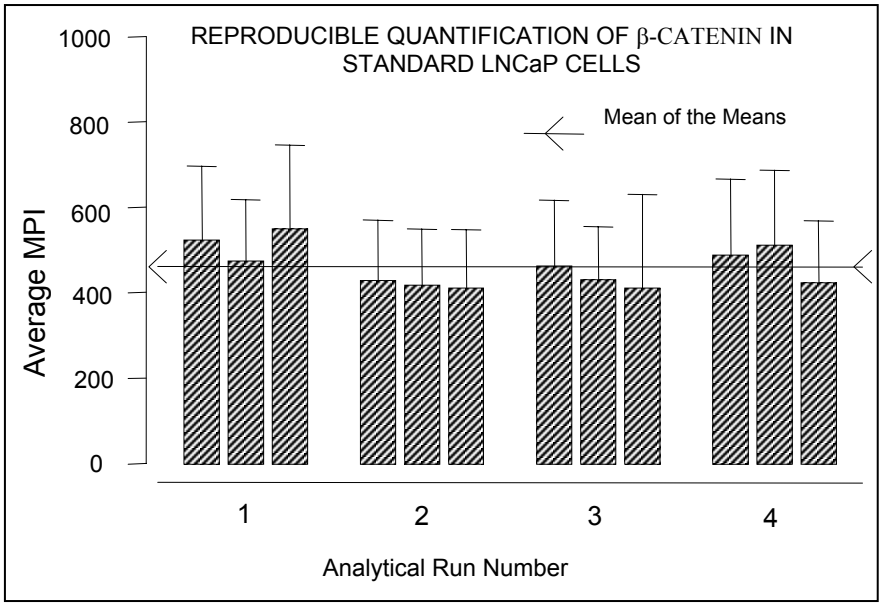

Figure 3. Triplicate slides of standard LNCaP cells were labeled with saturating dilutions of a mouse monoclonal anti-β-catenin antibody (Zymed) and an anti-mouse IgG-Alexa Fluor 568 conjugate, in four separate experiments. Fluorescence emission was determined as mean pixel intensity/cell (MPI). Each bar represents the average MPI ± standard deviation of 400-700 cells. Within each run, individual slides differed from the triplicate means by an average of 5% and across runs, the average MPIs of triplicate slides differed from the mean of the means by 7%.

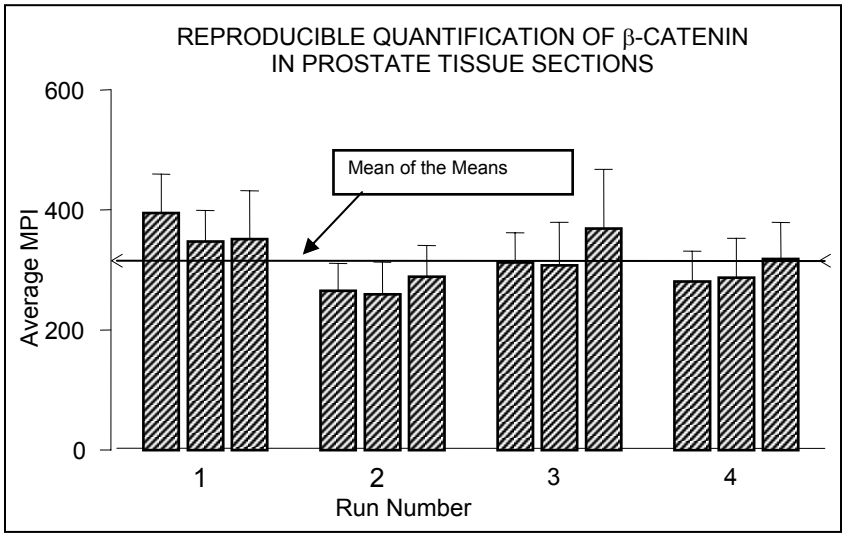

Figure 4. An archived, formalin-fixed specimen of a benign hyperplastic prostate gland was cut into 4-micron sections and mounted to glass slides. Slide specimens were treated for epitope recovery and labeled with saturating dilutions of mouse monoclonal anti-β-catenin antibody and a secondary goat anti-mouse IgG Antibody conjugated with Alexa Fluor 568. Fluorescence emission was determined as mean pixel intensity/acinus (MPI). Each bar represents the average MPI ± standard deviation of 100-200 peripheral zone acini. Within each run, individual slides differed from the triplicate means by an average of 6% and across runs, the average MPIs of triplicate slides differed from the mean of the means by 10%.

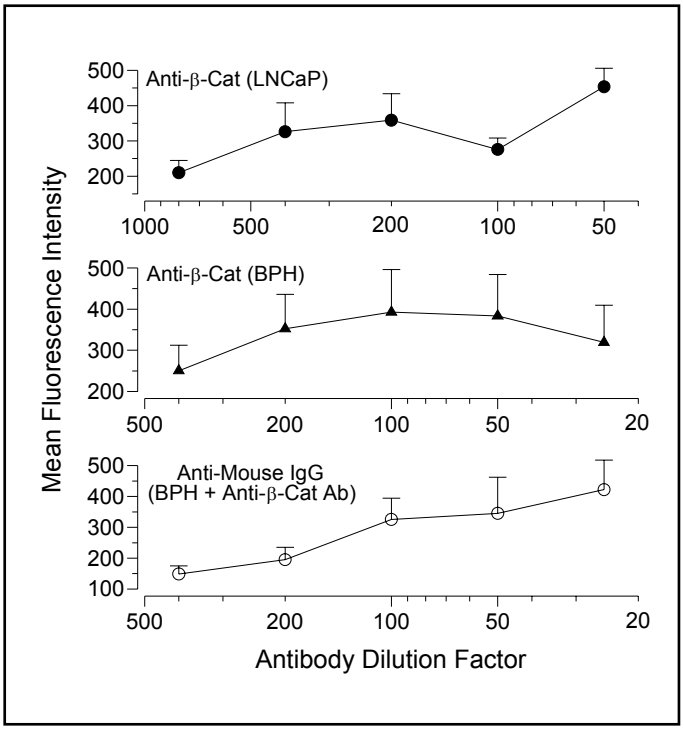

Figure 5: Titration of Primary and Secondary Antibodies for Stoichiometric, Fluorescence Labeling of β-Catenin in LNCaP Cells and Prostate Tissue Sections.

Primary anti-β-catenin Ab was titrated against β-catenin in LNCaP cells, establishing a dilution range that included a saturating dilution (1/200) at which stoichiometric labeling of the target protein is possible. Subsequently, the Ab was titrated against β-catenin in BPH tissue, over a range that included the LNCaP titration range. A saturating dilution of 1/100 was used for quantitative analysis of matched core biopsies. Similarly, secondary anti-mouse IgG-Alexa 568 was titrated against anti-β-Catenin-saturated sites in BPH tissue. A saturating dilution of 1/100 was used for quantitative analysis of matched core biopsies.

REPORTABLE OUTCOMES

Abstracts (Attached in Appendix)

1. Nizar K. Wehbi, George P. Casale, Dali Huang, Jun Tian, George P. Hemstreet III. Quantitative Fluorescence Imaging Analysis: an emerging technology for sensitive and reproducible proteometry with slide preparations of tissue sections and single cells. American Association for Cancer Research (AACR) 96th Annual Meeting, Anaheim, California, April 16-20, 2005.

2. Dali Huang, George P. Casale, Nizar K. Wehbi, Jun Tian, Neil A. Abrahams, George P. Hemstreet III. Quantitative Fluorescence Imaging Analysis of β-Catenin in normal appearing areas of prostate core biopsies: demonstration of a proteometric field change associated with prostate cancer. American Association for Cancer Research (AACR) 96th Annual Meeting, Anaheim, California, April 16-20, 2005.

3. Dali Huang, George P. Casale, Nizar K. Wehbi, Neil A. Abrahams, Jun Tian and George P. Hemstreet III. Quantitative Fluorescence Imaging Analysis for Detecting Cancer-Associated Changes in the Proteome of Morphologically Normal Acini in Cancerous Prostate Glands. South Central Section (SCS) of the American Urological Association, Inc. (AUA) 84th Annual Meeting, Austin, TX, September 15-18, 2005.

4. Dali Huang, Nizar Wehbi, Neil A Abrahams, Jun Tian, George Casale, George P. Hemstreet. Tissue Proteomics on Prostate Core Biopsy Specimens for Cancer Risk Assessment Using Quantitative Fluorescence Image Analysis. Fourth Annual AACR International Conference, Frontiers in Cancer Prevention Research. October 30-November 2, 2005. Baltimore, MD.

5. Dali Huang, George P. Casale, Nizar K. Wehbi, Jun Tian, George P. Hemstreet, III. Altered expression of cell adhesion molecules in normal appearing acini of cancer-bearing prostate glands, detected by Quantitative Fluorescence Imaging Analysis. 2006 Prostate Cancer Symposium by the American Society of Clinical Oncology (ASCO). February 24-26, 2006. San Francisco, CA.

6. Dali Huang, George P. Casale, Nizar K. Wehbi, Jun Tian, George P. Hemstreet, III. Altered expression of β-catenin and UDP-glucose dehydrogenase in normal appearing acini of cancer-bearing prostate glands, detected by Quantitative Fluorescence Imaging Analysis. 42nd ASCO Annual Meeting, June 2-6, 2006. Atlanta, Georgia.

Publications (Attached in Appendix)

1. Dali Huang, George P. Casale, Jun Tian, Nizar K. Wehbi, Neil A. Abrahams, Zahid Kaleem, Lynette M. Smith, Sonny L. Johansson, Johny E. Elkahwaji, and George P. Hemstreet III. Quantitative Fluorescence Imaging Analysis for cancer biomarker

discovery: Application to β-catenin in archived prostate specimens (In Press, July 2007, *Cancer Epidemiology Biomarkers & Prevention*).

Grants Submitted based on the Developed Methodology

Development of a Bladder Cancer Screening Test
Principal Investigator: George P. Hemstreet, III, M.D., Ph.D.
NIH – Phase I STTR
Submitted: 04/01/05
One year at $94,420 Direct Costs
SBIR/STTR Fee - $9,253
Goal: To develop a Bladder Cancer screening test for early detection and risk assessment of bladder cancer.

Translational Studies of Oxidative Stress in the Prostate, by Quantitative Fluorescence Imaging Analysis
Principal Investigator: George P. Casale, Ph.D
NIH RO1
Submitted: 10/01/05
Three years at $175,000/year
Goal: Using Quantitative Fluorescence Imaging Analysis, we will achieve quantitative analyses of oxidative damage and expression of key pro- and anti-oxidant enzymes in cancerous and non-cancerous prostate specimens retrieved from tissue archives, and we will compare these measurements, directly, in the same study. The work constitutes a quantitative translational study of oxidative mechanisms established in experimental systems.

CONCLUSIONS

There is a surprising disparity between the number of protein-encoding genes (*ca*. 30,000) in the human genome and the number of proteins (*ca*. 300,000) in the human proteome has inspired the development of translational proteomics aimed at protein expression profiling of disease states. Translational proteomics, which offers the promise of early disease detection and individualized therapy, requires new methods for analysis of clinical specimens. We have developed Quantitative Fluorescence Imaging Analysis (QFIA) for accurate, reproducible quantification of proteins in slide-mounted tissues. The method has been validated for analysis of β-catenin in archived prostate specimens fixed in formalin.

We fine-tuned and optimized the tissue fixation method, deparaffinization and epitope recovery procedures, and labeling procedures by using a two-antibody system. The use of ProLong® Gold antifade and a mercury/xenon (Hg/Xe) excitation lamp ensured higher protection against photobleaching. Intra-and inter-experimental reproducibility was confirmed and the Leica system tested before every use. β-catenin expression was analyzed in a cross-sectional case-control study that included 42 cancer cases and 42 controls matched on the basis of age (±5 years) and year of biopsy (±3 years).

In conclusion, we have developed tissue-based QFIA, a new method for quantification of proteins and secondary modifications of proteins in precisely selected elements of slide-mounted

specimens, in the context of observable histological features. We have validated by Reverse-Phase Protein Array Analysis (RPPA) the application of QFIA to β-catenin in archived prostate tissue fixed in formaldehyde-based fixative (r = 0.97).. Reduced expression of β-catenin in Normal Appearing Acini (NAA) relative to the Normal Acini (NA) of matched controls is a potential field marker for Prostate Cancer, in biopsies that miss existing adenocarcinomas. The observed sensitivity (42%) and specificity (88%) qualify the marker as a potentially significant contributor to a small panel of field markers, and support the feasibility of applying QFIA to the development of such a panel.

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Abstract Number:	411
Presentation Title:	Quantitative Fluorescence Imaging Analysis: an emerging technology for sensitive and reproducible proteometry with slide preparations of tissue sections and single cells
Presentation Start/End Time:	Sunday, Apr 17, 2005, 8:00 AM -12:00 PM
Category:	CH03-06 Proteomic analysis
Author Block:	*Nizar K. Wehbi*, *George P. Casale*, *Dali Huang*, *Jun Tian*, *George P. Hemstreet III*. University of Nebraska Medical Center, Omaha, NE

Transcriptome and proteome profiling have identified cancer-like changes in normal-appearing areas of cancerous prostate glands, providing a basis for developing procedures aimed at early cancer diagnosis and detection of premalignant disease. Comprehensive implementation of these goals will be achieved with technologies that allow sensitive measurements of subtle proteomic changes in cells and tissues. Quantitative Fluorescence Imaging Analysis (QFIA) is an emerging technology that allows sensitive and precise quantification of specific proteins in slide preparations of tissues and single cells. In this study, we examined conditions influencing sensitivity and reproducibility of QFIA applied to slide preparations of cancer cell lines and prostate tissue. Cancer cell lines cultured in the laboratory were fixed and imprinted to glass slides. Tissue slices of non-cancerous prostate glands collected by cystoprostatectomy were fixed in the laboratory and paraffin embedded. Four-micron sections were mounted to glass slides. Slide specimens were stoichiometrically labeled with 1^{o} antibody (Ab) and fluorophore-tagged 2^{o} Ab (Fl-Ab) or with 1^{o} Ab, biotinylated 2^{o} Ab and fluorophore-tagged streptavidin (Fl-SA). Labeling was done with a BioGenex *i*6000™ Autostainer and images of fluorescent events were captured with an automated Leica microscope fitted with a high-resolution CCD b/w camera. Captured images were segmented and fluorescence emission was quantified as mean fluorescence intensity (MFI), with Image-Pro® Plus software. In prostate specimens, the relative MFI of β-catenin was 100, 80, and 50 and the relative MFI of transglutaminase was 100, 70, and 0, after tissue fixation with zinc-based S.T.F.® Streck fixative, 5% formaldehyde, and Methacarn, respectively. Autostainer slide batching, which produced a 25% decline of β-catenin MFI in prostate specimens, was corrected by improvised programming. Incubation of slide specimens with 1^{o} Ab for 15-20 hours at 4^{o} C *vs.* 1 hour at 25^{o} C produced a 2-fold increase of thymosin-β-4 MFI, but did not increase β-catenin MFI. The signal:noise ratio of anti-gelsolin-labeled cell lines was increased from 3:1 to 12:1 by labeling with Fl-Ab *vs.* Fl-SA, while background corrected signals were nearly the same. Photobleaching and storage-dependent signal loss were obviated, by mounting specimens in ProLong® Gold antifade *vs.* N-propyl gallate or *SlowFade*® Antifade. Standard cell lines incorporated into each analysis and imaging system assessment with InSpeck™ Green Calibration fluorescent beads served to establish reproducible measurements. In conclusion, by fine-tuning our QFIA procedures, we have achieved intra- and inter-experiment and person-to-person reproducibility of ± 10% and can readily detect a 20% difference in tissue or single-cell protein expression.
[DoD grant DAMD17-02-1-0121]

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Abstract Number:	777
Presentation Title:	Quantitative fluorescence imaging analysis of β-Catenin in normal appearing areas of prostate core biopsies: Demonstration of a proteometric field change associated with prostate cancer
Presentation Start/End Time:	Sunday, Apr 17, 2005, 8:00 AM -12:00 PM
Category:	PR01-04 Biomarkers and intervention studies
Author Block:	*Dali Huang*, *George P. Casale*, *Nizar K. Wehbi*, *Jun Tian*, *Neil A. Abrahams*, *George P. Hemstreet III*. Univ. of Nebraska Medical Ctr., Omaha, NE

Measurement of serum PSA remains the principal tool for prostate cancer (PC) screening. A PSA test of 4-10 ng/mL is associated with early-stage, highly treatable PC, but exhibits a specificity of only 25%, requiring biopsy for diagnosis. Biopsy, however, misses tumor nodules and includes only normal-appearing areas (NAA) in 20% of cases, necessitating rebiopsy of the large majority of these patients. The principal objectives of this work were to determine whether cancer-associated field events may be detected as proteometric changes of the cell adhesion molecule β-catenin in NAA of cancer-bearing prostate glands, and to evaluate proteometric changes of β-catenin in NAA, as a potential biomarker of PC. H&E-stained slide specimens of archived core biopsies from PC cases and benign prostatic hyperplasia (BPH) controls were screened for intact tissue architecture. Fourteen sets of cases and controls, matched one-to-one on the basis of patient age and year of biopsy, were included in this pilot study. Duplicate slide-mounted specimens were stoichiometrically labeled with anti-β-catenin antibody and fluorophore-conjugated secondary antibody. Twelve-bit gray scale images were captured with a fully automated Leica microscope fitted with a mercury/xenon excitation lamp and a high-resolution CCD b/w camera. Images were segmented and fluorescence emission was quantified with ImagePro Plus software, as mean fluorescence intensity of individual prostatic acini (MFI), and the average MFI (AMFI) of 40 to 200 acini per core was determined. The AMFI in NAA of five cases (634 ± 167) was significantly (p=0.03) greater than the AMFI of the matched controls (454 ± 57), whereas the AMFI in NAA of the remaining nine cases (388 ± 147) was significantly (p=0.003) lower than the AMFI of the matched controls (562 ± 115). Two of the nine cases under-expressing β-catenin in NAA had AMFIs (291 ± 99 and 217 ± 128) well below the lowest AMFI (405 ± 102) of the fourteen BPH controls. In conclusion, dysregulated phenotypic expression of β-catenin has been identified as a cancer-associated field event in NAA of core biopsies. Further, an AMFI of *ca.* 300 or less for β-catenin in normal-appearing core biopsies may detect one in seven PCs. Funded by DoD grant DAMD17-02-1-0121.

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QUANTITATIVE FLUORESCENCE IMAGING ANALYSIS FOR DETECTING CANCER-ASSOCIATED CHANGES IN THE PROTEOME OF MORPHOLOGICALLY NORMAL ACINI IN CANCEROUS PROSTATE GLANDS

Dali Huang, George P. Casale, Nizar K. Wehbi, Neil A. Abrahams, Jun Tian and George P. Hemstreet III

Introduction: The study objective is to develop a quantitative tissue-based assay for proteomic changes in morphologically normal acini of cancer-bearing prostate glands (CBP), which define individual risk of prostate cancer (PC). The work is predicated on previous studies of bladder cancer (*Proc. Natl. Acad. Sci. USA* 90:8287, 1993) and is based on the hypothesis that normal-appearing areas of CBP express proteomic changes representing premalignancy (field disease) or a response to cancer (field effect).

Methods: Archived specimens of non-cancerous prostate glands (BPH) and CBP were age-matched and matched to year of collection. Specimens were stoichiometrically labeled (BioGenex autostainer) with primary antibodies to tissue Transglutaminase (tTGase) or beta-Catenin (b-CAT) and fluorophore-conjugated secondary antibody. Fluorescence images were captured with a CCD camera, Leica automated microscope fitted with an Hg/Xe lamp, and ImagePro software. Signals from single cells and prostatic acini were digitized on a 12-bit gray scale.

Results: QFIA conditions were optimized to intra- and inter-assay signal variations of less than 10% for standard tissues and cell lines. The optimized protocol, which discriminated single cell expression differing by 20%, was applied to tTGase and b-CAT expression in prostatic acini. TTGase was reduced ($P<0.05$) in cancerous acini (N=166; mean of 413) and normal-appearing acini (N=356; mean of 424) of CBP (6), compared to normal acini (N=272; mean of 541) of BPH (6). However, variable expression in normal-appearing acini of CBP and normal acini of BPH disqualified the protein for individual risk assessment with core biopsies. In contrast, b-CAT expression was homogenous in acini of BPH and qualified as a potential biomarker for core biopsies. Mean fluorescence signal (MFS) in normal acini (N=1477; mean of 221) of BPH (10) and normal-appearing acini (N=1285; mean of 223) of CBP (10) was different ($P<0.05$) from cancerous acini (N=2007; mean of 126). Scatter plots suggest 20-30% of normal-appearing acini in CBP will reproducibly fall below a threshold separating them from BPH acini.

Conclusions: We have for the first time developed instrumentation and methods for quantitative analysis of proteins in tissue sections. Utilizing this technology, we identified b-CAT as a candidate for a biomarker panel for determining risk of prostate cancer in individuals with an elevated PSA and a negative biopsy.

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compounds were added to extracts of the tissues, and the products were purified by chromatography. Conversion of E1 to ES in normal and tumor tissues was: N=7, 0.73 ± 0.25 and 0.88 ± 0.32 % of the dose/h/g in the presence of estrone sulfamate. In contrast, sulfatase was highly active in normal and tumor tissues: N=7, 47.4 ± 13.2 and 70.8 ± 13.2 %/h/g (6.5 ± 1.9 and 8.7 ± 1.4 pmol/h/g), respectively, and was not significantly greater in the tumor tissue. Conversion of E1 to E2 was less in normal tissue than in tumors (N=5, 0.24 ± 0.12 and 10.4 ± 9.8 pmol/h/g, respectively), with great variability among tumors. The reverse reaction was greater in the normal tissue, N=3, 6.7 ± 5.5 and 0.40 ± 0.16 pmol/h/g, respectively. It is concluded that availability of estrone sulfate is similar in normal and tumor tissues but that in some tumors 17β-HSD activity is greatly increased and contributes, with increased aromatase activity, to the estrogenic stimulation of ER positive tumors.

C12 Glutathione S-Transferase T1, M1 Genetic Polymorphisms in Cases of Acute Leukemia. Magdy M. El-Bordiny,[1] Ashraf H. Ghandour,[2] Manal El Sorady,[2] Shaden M. Hanafi,[3] Abir A. Saad.[4] Department of Clinical Pathology, Faculty of Medicine, Alexandria University,[1] Alexandria, Egypt, Department of Internal Medicine, Faculty of Medicine, Alexandria University,[2] Alexandria, Egypt, Genetic Engineering Institute,[3] Menoufia University, Egypt, Institute of Graduate Studies and Research - Alexandria University,[4] Alexandria, Egypt.

Glutathione S-transferases (GSTs) are enzymes that detoxify potentially mutagenic and carcinogenic xenobiotics. The genes encoding isoenzymes M1 and T1 are polymorphic in humans and the phenotypic absence of enzyme activity is caused by a homozygous inherited deletion of the gene. The null genotype has been associated with incidence of several types of solid tumors. In addition, GSTM1 and GSTT1 polymorphisms have been considered as risk factors for developing acute leukemia in a number of studies; however the overall results of these previous reports are inconsistent and even controversial. This study was undertaken to determine the frequencies of allelic variants of the GST genes in Egyptians and to explore the possible influence of GSTT1, M1 genetic polymorphisms on the risk of acute leukemia (myeloid and lymphoid). We have determined the prevalence of the GSTM1 and GSTT1 homozygous deletions in 55 Egyptian patients with leukemia (21 ALL and 34 AML) and 87 racially, geographically, age and sex matched healthy control individuals using a multiplex polymerase chain reaction (PCR)-based methods. The genotype frequencies in the leukemia patients and normal controls were compared using Fisher s exact test. A significantly increased incidence of the GSTT1 null genotype was found in the group of patients compared to the controls (34% versus 15% P = 0.03, OR = 2.98, 95% CI 1.6-7.6). GSTM1 null genotype frequencies in cases were slightly but non-significantly higher than controls (68 % versus 49 %, respectively, P=0.07). Stratification with leukemia immunophenotypes was made for further comparison. This study will be further extended to examine the relation between the GSTT1 and GSTM1 null genotypes and the patient s response to therapy.

C13 Aromatase Gene Polymorphism and Age at Diagnosis in Ovarian Tumors of Low Malignant Potential. Mark I. Hunter, David Peel, Wendy R. Brewster. University of California, Irvine, Irvine, California.

Objective: Recent studies have demonstrated an association between womens cancer, including breast and uterine cancer, and the presence of polymorphisms in the genes that control endogenous estrogen biosynthesis. We evaluated the presence of the TTTA microsatellite polymorphism in the aromatase (CYP19) gene and the age at diagnosis in a group of patients with ovarian tumors of low malignant potential (LMP). Methods: The study was conducted based on 68 patients with LMP tumors, recruited from the regional tumor registry. Individuals participated in a telephone interview, followed by the submission of a genetic sample either by cheek swab or by blood sample. Patients were then stratified by the number of TTTA repeats. Association with age at diagnosis was then determined using Wilcoxon rank sum and linear regression. Results: Of the 68 patients studied, 58 were found to have at least one CYP19 allele with 7 TTTA repeats, while 19 of these had 7 TTTA repeats on both alleles. This was the shortest allele repeat identified in the present study. Four patients had an 8 TTTA repeat, one had a 9-repeat, and four had 11 repeats as their shortest allele. The mean age at diagnosis of an LMP tumor was noted to be 45 years for patients with the presence of a 7-TTTA repeat and 53 years old for those who did not have a 7 repeat. The difference was not significant. Conversely, patients who had 12-repeats in their longest allele had a median age at diagnosis of 65, compared to 46 years old for patients who did not have the longest allele (p=0.02). Using regression analysis, the length of the shortest allele was directly correlated with an older age at diagnosis (p=0.04). Conclusion: The age at diagnosis for ovarian tumors of low malignant potential appears to be correlated with the presence of polymorphisms in the aromatase gene, with an older age at diagnosis associated with more TTTA repeats. There is evidence to suggest that longer TTTA repeats are associated with estrogen sensitive tumors, such as breast and uterine cancer. Perhaps there is a protective effect against ovarian tumors from the increased aromatase activity in individuals with longer repeats, such as a relative suppression of ovulation. This would be consistent with the finding that aromatase inhibitors are potent agents of ovulation induction. This is a small study, and the results should be interpreted with caution while we await larger analyses.

C14 Tissue Proteomics on Prostate Core Biopsy Specimens for Cancer Risk Assessment Using Quantitative Fluorescence Image Analysis. Dali Huang, Nizar Wehbi, Neil A Abrahams, Jun Tian, George Casale, George P. Hemstreet. University of Nebraska Medical Center, Omaha, Nebraska.

Purpose: The purpose of this project was to develop a tissue proteomic biomarker assay for human biopsy specimens and to utilize the quantitative fluorescence image analysis (QFIA) assay to detect individuals with an elevated PSA at risk for prostate cancer. The concept for this objective builds on previous experiments and clinical trials aimed at detecting premalignant field disease biomarker(s) on single cells and the clinical screening of individuals at risk for bladder cancer. Specifically, the objective of this project is to develop a biomarker profile to detect biologically active prostate cancer in individuals with an elevate PSA and or prostate nodule who are initially biopsy negative. Method: Prostate core biopsy specimens were obtained for 40 patients with prostate cancer and from 40 matched (±5 years) controls biopsied in the same year who did not develop prostate cancer within 5 to 10 years. Specimens were stoichiometrically labeled (Biogenex Stainer) with primary antibodies to β-Catenin and a fluorphore conjugated secondary antibody (Alexa Fluor 488). Fluorescence images were captured with the Hamamatsu Digital b/w Camera, Leica DMXRA2 automated microscope fitted with a Hg/He lamp, and Image-Pro Plus Software (Meyer Instruments). Signals from single cells and prostate acini were digitalized on a 12-bit gray scale. Results: QFIA instrumentation calibration was achieved with fluorescence beads and LNCaP cell line and normal prostate peripheral zone tissue. The average mean fluorescence intensity for the duplicate runs for the cases and controls was plotted as a ROC determination. The specificity was selected for 100%. Under these condition 28% of the prostate cancers were detected in the premalignant field based on abnormal expression of β-catenin at a threshold set below 500 MFI. Utilizing a students t test there was a differential level of biomarker expression amongst the cancer area and the normal appearing acini in the cancer gland compared to the normal control ($p<0.01$). In a matched pair analysis there was also a statistically significant difference between the normal appearing areas in the prostate gland with cancer and the normal peripheral zone acini without cancer ($p<0.01$).Conclusion: Commercially available instrumentation was programmed for the detection of molecular proteomic fingerprints in normally appearing acini in core biopsies from individuals with biologically active prostate cancer. β-catenin is uniformly expressed in normal acini cells of the peripheral zone in individuals who are prostate cancer free for 5 years following the initial biopsy, a requisite for minimal sample analysis. Utilizing the QFIA technology we identified β-catenin as a candidate biomarker for a profile to determine risk of prostate cancer in individuals with an elevated PSA and a negative biopsy.

C15 Repeatability and Long Term Stability of Ki-67 Immunostaining, Cell Numbers and Cytologic Morphology in Random Periareolar Fine Needle Aspiration Samples of Breast Tissue Processed as Thin Layer Preparations. Brian K. Petroff,[1] Trina Metheny,[1] Qiao Xue,[1] Kelli E. Valdez,[1] Carola M. Zalles,[2] Bruce F. Kimler,[1] Carol J. Fabian.[1] Univ. of Kansas Medical Ctr.,[1] Kansas City, KS, Yale University,[2] New Haven, CT.

Introduction: Random periareolar fine needle aspiration (RPFNA) is a minimally invasive technique permitting serial assessment of tissue based biomarkers during breast cancer prevention trials. Commonly used breast biomarkers include proliferation (assessed by Ki-67 immunostaining) and cytologic morphology. Previous studies have associated hyperplasia with atypia on RPFNA cytomorphology with a 5-fold increase in the risk of breast cancer in a high risk cohort. This work was performed to assess the robustness of these markers through evaluation of both the immediate repeatability and long term stability of breast RPFNA samples as reflected in cytology and Ki-67 expression. **Materials and Methods:** Benign breast tissue cytology specimens obtained by RPFNA from high risk women were processed as thin layer preparations using a Cytyc 2000 platform. RPFNA samples were fixed overnight in modified Cytolyt (9 ml Cytolyt + 1 ml 10% neutral buffered formalin) and stored in methanol-based PreservCyt solution. Ki-67 staining was performed using antigen retrieval in citrate buffer at 125oC, quenching of endogenous peroxidase followed by incubation with primary antibody (mouse-anti-human Ki67, Dako clone MIB-1, 1:20 dilution), secondary antibody labeling and color reaction. Blank diluent was used as negative control. Both Ki-67 immunoreactivity (% immunopositive cells) and cytologic morphology were evaluated

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Altered expression of cell adhesion molecules in normal appearing acini of cancer-bearing prostate glands, detected by quantitative fluorescence imaging analysis.

Sub-category: Diagnosis, Staging and Treatment
Category: Early and Localized Disease
Meeting: 2006 Prostate Cancer Symposium

Abstract No: 56

Author(s): D. Huang, G. P. Casale, N. K. Wehbi, J. Tian, G. P. Hemstreet

Abstract: **Introduction:** Expression of cell adhesion molecules including β-catenin, annexin I, and connexins 43 and 32 is markedly altered in prostate adenocarcinomas. The objective of this work was to evaluate expression of these proteins in normal appearing acini (NAA) of cancer-bearing prostate glands, as potential biomarkers of PC field disease. **Methods:** β-Catenin, in archived core biopsies of 42 pairs of PC cases and controls, and annexin I and connexins 43 and 32, in a tissue microarray of 10 pairs of cases and controls, were labeled with primary antibodies and fluorophore-conjugated secondary antibodies. Fluorescence in prostatic acini was measured with an automated Leica microscope and Image Pro Plus software and expressed as mean pixel intensity (MPI) per acinus and average MPI (AMPI) per case or control. **Results:** Mean AMPI of β-catenin in NAA of 33 cases (545 ± 93) was lower ($p<0.01$) than that for normal acini (NA) of the age-matched controls (684 ± 90), whereas mean AMPI for NAA of the remaining 9 cases (712 ± 111) was higher ($p=0.003$) than that of the matched controls (634 ± 95). Twelve cases under-expressing β-catenin in NAA had AMPIs below the lowest AMPI (506 ± 83) of all controls. Connexin 43 signal was observed only in the basal cell layer. Two cases over-expressing connexin 43 in NAA had AMPIs above the highest AMPI (207 ± 40) of all controls. Three cases under-expressing connexin 43 in NAA had AMPIs below the lowest AMPI (112 ± 43) of all controls. Expression of connexin 32 and annexin I in NAA and NA was not significantly different. **Conclusions:** Reduced expression of β-catenin is a cancer-associated event in NAA. A cut-off AMPI (506) for β-catenin detected 29 % of PC cases with 100% specificity. Altered expression of connexin 43 in NAA is a potential biomarker of PC. An upper cut-off AMPI (207) and a lower cut-off (112) identified 50% of PC cases with 100% specificity. Funded by DoD grant DAMD17-02-1-0121.

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Altered expression of β-catenin and UDP-glucose dehydrogenase in normal appearing acini of cancer-bearing prostate glands, detected by quantitative fluorescence imaging analysis.

Sub-category: Prostate Cancer
Category: Genitourinary Cancer
Meeting: 2006 ASCO Annual Meeting

Abstract No: 14540
Citation: *Journal of Clinical Oncology*, 2006 ASCO Annual Meeting Proceedings Part I. Vol 24, No. 18S (June 20 Supplement), 2006: 14540

Author(s): D. Huang, G. P. Casale, N. K. Wehbi, J. Tian, G. P. Hemstreet

Abstract: **Background:** Expression of β-catenin and UDP-glucose dehydrogenase (UGDH) is markedly altered in prostate adenocarcinomas. The objective of this work was to evaluate expression of these proteins in normal appearing acini (NAA) of cancer-bearing prostate glands, as potential biomarkers of PC field disease. **Methods:** Archived core biopsies of 42 pairs of PC cases and controls were labeled for β-Catenin, and a tissue microarray of 10 prostatectomy specimens of cancer cases and controls were labeled for UGDH. A fluorophore-conjugated secondary antibody was incorporated into the staining protocol. Fluorescence in prostatic acini was measured with an automated Leica microscope and Image Pro® Plus software and expressed as mean pixel intensity (MPI) per acinus and average MPI (AMPI) per case or control. **Results:** Mean AMPI of β-catenin in NAA of 33 cases (545 ± 93) was lower ($p < 0.01$) than that of normal acini (NA) in age-matched controls (684 ± 90), whereas mean AMPI of NAA in the remaining 9 cases (712 ± 111) was higher ($p = 0.003$) than that of the matched controls (634 ± 95). Twelve cases under-expressing β-catenin in NAA had AMPIs below the lowest AMPI (506 ± 83) of all controls. Mean AMPI of UGDH in NAA of 7 cases (372 ± 169) was lower ($p < 0.05$) in comparison to both NA (558 ± 119) and cancerous acini (CA) (645 ± 88). The difference between CA and NA did not achieve statistical significance. **Conclusions:** Reduced expression of β-catenin is a cancer-associated event in NAA. A cut-off AMPI (506) for β-catenin detected 29% of PC cases with 100% specificity. Altered expression of UGDH in NAA is a potential biomarker of PC. Funded by DoD grant DAMD17-02-1-0121.

QUANTITATIVE FLUORESCENCE IMAGING ANALYSIS FOR CANCER BIOMARKER DISCOVERY: APPLICATION TO β-CATENIN IN ARCHIVED PROSTATE SPECIMENS

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ABSTRACT

The surprising disparity between the number of protein-encoding genes (*ca*. 30,000) in the human genome and the number of proteins (*ca*. 300,000) in the human proteome has inspired the development of translational proteomics aimed at protein expression profiling of disease states. Translational proteomics, which offers the promise of early disease detection and individualized therapy, requires new methods for analysis of clinical specimens. We have developed Quantitative Fluorescence Imaging Analysis (QFIA) for accurate, reproducible quantification of proteins in slide-mounted tissues. The method has been validated for analysis of β-catenin in archived prostate specimens fixed in formalin.

QFIA takes advantage of the linearity of fluorescence antibody signaling for tissue epitope content, a feature validated for β-catenin in methacarn-fixed prostate specimens analyzed by reverse-phase protein array analysis (RPPA) and QFIA ($r = 0.97$). QFIA of β-catenin in formaldehyde-fixed tissues directly correlated with β-catenin content ($r = 0.86$). Application of QFIA in a cross-sectional study of biopsies from 42 prostate cancer (PC) cases and 42 matched controls, identified β-catenin as a potential, field marker for PC. Receiver operating characteristic plots revealed that β-catenin expression in the normal-appearing acini of cancerous glands identified 42% (95% C.I., 26%-57%) of cancer cases, with 88% (95% C.I., 80%-96%) specificity. The marker may contribute to a PC biomarker panel. In conclusion, we report the development and validation of a new method for fluorescence quantification of proteins in archived tissues and its application to archived specimens for an evaluation of β-catenin expression as a biomarker for PC.

INTRODUCTION

The availability of structural and functional data for thousands of cellular proteins (1) has inspired wide-spread interest in translational studies aimed at protein expression profiling of clinical diseases and their antecedent states. A key to implementation of this translational research is the development of techniques that offer sensitive, continuous quantification of proteins in clinical specimens. Gene microarray studies have stimulated development of protein profiling techniques such as protein microarray analysis (2), biological mass spectrometry (3) and tissue-based fluorescence analysis (4, 5), which are applied to small, tissue samples, ***e.g.***, biopsies. The application of gene microarrays to molecular profiling of cancers provided the first glimpses into the remarkable molecular heterogeneity of cancer among patients and among tumors in the same patient, and identified cancer subclasses defined by common expression patterns (6). In relation to cancer protein profiling, gene microarrays have identified numerous proteins whose expression may be modified (7-10). Protein networks directly carry out cellular functions and dysregulation of these networks *via* altered protein expression or post-translational modification underlies disease.

Reverse-phase protein array analysis (RPPA) (2, 6, 11-13) and automated quantitative analysis (AQUA) (4, 5, 14) are two recently introduced methodologies that precisely quantify proteins and their secondary modifications in clinical specimens, ***i.e.***, slide-mounted tissues. RPPA was developed with a focus on modifications of cell-signaling cascades in disease, and simultaneously analyzes multiple lysates of specimens from one or more patients. Nanoliter volumes of lysate are arrayed onto nitrocellulose slides and probed with a primary antibody detected with a fluorophore-conjugated secondary antibody. Protein array analysis exhibits high sensitivity, precision, and linearity across a wide range of protein concentrations. AQUA was developed with a primary focus on rapid and precise quantification of protein expression in tissue microarrays for large-scale studies of disease outcome. AQUA entails mapping sub-

cellular compartments of tissue specimens by fluorescence antibody labeling and assignment of the fluorescence signal from biomarkers of interest, to specific compartments. The assay has produced remarkable improvements over pathologist classification of immunohistochemical (IHC) staining, in the prediction of population-based, disease outcomes. The present study introduces a novel quantitative fluorescence imaging analysis (QFIA) procedure for rapid and precise quantification of proteins and their secondary modifications, in the context of conventional, histological features of slide specimens. The procedure complements RPPA and AQUA.

Diagnosis of prostate cancer (PC), the second leading cause of cancer deaths among U.S. men (15), is dependent on quantification of serum PSA. A PSA test of 4-10 ng/mL, however, has a specificity of only 25% (16) and requires biopsy, which has a false negative rate of 20% (17), necessitating rebiopsy of the large majority of these men. A potential solution to this problem is based on the concept of biomarker alterations associated with premalignant field disease and cancer-induced field effect (18-20). Premalignant field disease is defined as genetic and/or epigenetic modifications of morphologically normal-appearing cells that herald an increased probability of neoplastic transformation (18, 20). In response to continued exposure to carcinogenic insult, cells at multiple foci are initiated (polyclonal disease) and undergo progression toward PC (19). In contrast, field effect is defined as the presence of epigenetic changes in cells outside of the tumor nodules, in response to altered signaling from cancer cells (18, 20). Cells within the premalignant field or the area of field effect exhibit a normal morphology but express phenotypic modifications that may be detected as qualitative or quantitative biochemical changes (18, 20). Consequently, it may be possible to identify patients with early cancer on the basis of a positive cancer biopsy or a morphologically normal-appearing biopsy expressing biochemical field disease/effect.

An objective of the present study was to evaluate the adhesion/signaling protein β-catenin as a potential field marker for prostate cancer. β-Catenin is a 92-kD protein encoded by a gene localized to human chromosome 3p22 (21). The protein expresses both cell adhesion and transcriptional regulation functions that are independent of each other. Impaired β-catenin degradation with increased cellular concentration of the protein, contributes to inappropriate activation of transcription factors and uncontrolled cell proliferation. Transmembrane cadherin molecules, the prime mediators of calcium-dependent cell-cell adhesion, express an intracellular catenin-binding domain (22). Binding of β- or γ-catenin to this domain is required for linkage of E-cadherin to actin filaments of the cytoskeleton. Deletion of the catenin-binding domain of E-cadherin or functional inactivation of catenins disables cell-cell adhesion even though E-cadherin retains its extracellular binding domain. In the prostate, β-catenin appears to act primarily as a cell adhesion molecule rather than a transcription factor (23). The cell-cell adhesion complex is compromised in cancer-bearing prostate glands, as evidenced by marked down regulation of cadherins in the cancerous epithelium (24).

Work presented in this report addressed two, integrated objectives; 1) validation of a new method, tissue-based QFIA, for fluorescence quantification of β-catenin in slide specimens of archived prostate tissues that were fixed in formaldehyde-based fixative, and 2) evaluation of β-catenin expression as a potential field marker for prostate cancer, using archived prostate tissue specimens.

METHODS

Cells and Tissues

Archived core biopsies. The surgical pathology database of the Veterans Affairs Hospital at Omaha (VAHO) was searched for archived cancerous core biopsies and non-cancerous biopsies of patients who were followed for at least five years and remained without clinical PC. Patients without clinical cancer (controls) were matched one-to-one with cancer patients on the basis of age (± 5 years) and year of biopsy (± 3 years). The latter was expected to minimize potential, analysis variability due to differences in tissue fixation conditions or storage time. Selection of patients without clinical cancer was random with respect to non-cancerous prostate conditions, *i.e.*, no effort was made to select a particular non-cancerous condition, *e.g.*, prostatitis or benign prostatic hyperplasia (BPH). All procedures were in compliance with human studies protocols approved by the Institutional Review Board (IRB) of the institution. Fifty matched pairs were identified and their paraffin blocks were retrieved from the archives. All tissue cores had been fixed in 10% neutral buffered formalin, under unspecified conditions. Slides were prepared with 4-micron sections of each block containing 3-6 cores and then one slide per paraffin block was stained with hematoxylin and eosin (H&E). Two pathologists (N.A.A. and D.H.) evaluated the H&E-stained slide specimens, to determine suitability of each specimen for the study. The criteria for inclusion of a PC specimen were 1) the presence of both non-cancerous and cancerous epithelium, 2) a minimum of *ca.* 30 non-cancerous acini (the large majority of specimens contained 70-100 acini), 3) cancerous areas representing at least 25% of the biopsy specimen, and 4) cancerous epithelium representing *ca.* 80% or more of the cancerous areas. The pathologists identified two types of specimens from patients without clinical cancer, for the present study: those characterized by glandular hyperplasia (19 specimens) and those characterized as having no apparent pathologic lesions (23 specimens). Each of these specimens

contained from 30 to more than 200 acini, with the large majority containing 70-100 acini. Damaged specimens exhibiting cracks, fissures or fragmentation or poor H&E staining were excluded from the study because of the possibility of labeling artifacts. Excluded specimens represented no more than 2-3% of the specimens retrieved from the archive. Clinical and demographic data, excluding patient identifiers, were obtained from patient records and associated with each anonymous specimen.

Core biopsies from forty-two PC cases and matched controls were included in the present study for analysis of β-catenin expression. Clinical and demographic data are summarized in **Table 1**. At biopsy, the mean age of the cases was 67 years (range: 55-77 years) and the mean age of the controls was 67 years (range: 57-78 years); the age difference for each matched pair did not exceed five years. Biopsies were taken in the interval from 1991 to 1998; the difference for year-of-biopsy of each matched pair did not exceed three years. All control subjects were considered to be without clinical cancer at the time of biopsy because clinical follow-up including PSA determination five to eleven years later was negative for PC.

Prostatectomy specimens of consented subjects, for evaluation of tissue fixation procedures and for preparation of analytical standards. In compliance with IRB-approved protocols, tissue sections and fresh biopsy cores were collected from prostate glands of consented subjects who had undergone prostatectomy at the University of Nebraska Medical Center (UNMC) or the VAHO. Tissue specimens were collected from two benign hyperplastic (BH) glands characterized by glandular hyperplasia and one cancer-bearing gland to evaluate different fixation procedures for QFIA, according to a standard operating procedure established by the research laboratory. Tissue blocks from one BH gland were used as standards in each analytical run. Tissue sections of 200-300 mg were taken from the right and left sides of the gland and transferred directly into fixative. Sections were fixed with 4% EM grade formaldehyde, zinc-based Streck Tissue Fixative® (Streck Laboratories, Inc., LaVista, NE), or

methacarn fixative (25) for 12, 24, 48, and 72 hours. Tissues were embedded in paraffin, sectioned at 4 microns, mounted to glass slides and processed for analysis of the β-catenin fluorescence signal. Optimum fixation times yielding the brightest fluorescence signal were 24 hours for Streck fixative and 48 hours for formaldehyde and methacarn. Slide specimens of one BH gland fixed for 48 hours in 4% formaldehyde were used as analytical standards.

Serial sections of prostatectomy specimens for comparison of β-catenin expression determined by quantitative fluorescence imaging analysis (QFIA) and reverse-phase protein analysis (RPPA). Validation of quantitative imaging analysis of β-catenin in 4-micron sections of prostatectomy specimens fixed in methacarn was implemented with specimens from two BPH cases with glandular hyperplasia and eight cases of PC. These previously screened cases were selected to provide a wide range of β-catenin expression. Serial sections of each specimen were mounted to glass slides and pairs of adjacent sections were selected for study. One section of each pair was analyzed by QFIA and the matching section was analyzed by RPPA. A more distant section of each case served as an isotype control for QFIA.

A tissue microarray (TMA) for comparison of β-catenin expression in methacarn-fixed and formaldehyde-fixed tissues, determined by quantitative fluorescence imaging analysis (QFIA). A tissue Microarray was prepared with sections taken from the prostate glands of three BPH cases with glandular hyperplasia and seven PC cases. Two sections were collected from each gland; one section was fixed in methacarn and the second was fixed in 4% EM grade formaldehyde (as described above). All sections were embedded in paraffin and 1.0 mm cores were punched and transferred to the blank, paraffin block. Two cores each were taken from acini-rich areas, when possible, of formaldehyde-fixed and methacarn-fixed sections of each BH gland. For the cancer cases, pairs of cores were taken from areas rich in cancerous epithelium and from areas rich in non-cancerous acini, again, when possible. The TMA block was sectioned at 4 microns and mounted to glass slides. Expression of β-catenin in the tissue disks of duplicate

slide preparations was analyzed by QFIA. The array permitted direct, cross-fixative comparisons of the acini of the two BH glands, the non-cancerous acini of five cancer-bearing glands, and the cancerous epithelium of one cancer-bearing gland.

LNCaP cell standards for quantitative analysis. LNCaP cells (clone FGC of unknown passage) purchased from the American Type Culture Collection (Manassas, VA) were cultured in RPMI medium supplemented with 10% heat-inactivated bovine serum and passaged every 5-7 days. Cells at passage 10-15 were cryopreserved in Origen Freeze Medium (Fisher Scientific, Hampton, NH). Cryopreserved cells were seeded to T-flasks, grown to 70% coverage, harvested, washed in Dulbecco's phosphate buffered saline w/ Ca^{+2} Mg^{+2} (PBS), and suspended to 5 x 10^4 cells/mL PBS. Higher cell concentrations were associated with clumping during fixation and yielded poor slide specimens. EM grade formaldehyde (Polysciences, Inc., Warrington, PA) was added to 0.5% and the suspension was fixed for 15 min at room temperature. Subsequently, the suspension was diluted with an equal volume of QFIA FIXIT (26), held overnight at 4^o C and then dispensed to 4-mL cryo-vials and stored at -80^o C. One to three days prior to fluorescence labeling, cryopreserved suspensions were thawed and captured to 25 mm polycarbonate filters (Nucleopore; 5 micron pores; Fisher Scientific), with a glass filtration unit (Fisherbrand®; Fisher Scientific). The captured cells were washed with cell adherent fluid followed by modified Saccomanno Fixative and then blotted to OptiPlus™ barrier slides (BioGenex Laboratories, San Ramon, CA). Blotted cells were sprayed with Carbofix-E™ (StatLab Medical Products, Lewisville, TX), dried for 15 min, and the slides were stored at -20^o C for at least one day and not more than two weeks, prior to use.

Reverse-Phase Protein Analysis (RPPA)

Analysis of slide-mounted prostate specimens was implemented as described by Paweletz *et al.* (11). Slide specimens to be extracted were stained with Mayer's hematoxylin and reviewed by light microscopy for structural integrity. The entire section was shaved off the glass slide, with a single-edge razor blade, and deposited into 200 uL of extraction buffer consisting of a 1:1 mixture of 2x Tris-Glycine SDS sample buffer and Tissue Protein Extraction Reagent (TPER; Pierce Biotechnology, Rockford, IL) plus 2.5% β-mercaptoethanol. The sample was heated for two hours at 70^{o} C in a heat block (Eppendorf Thermomixer 5436) and then sonicated (Branson 2110) for ten minutes and heated at 95^{o} C for eight minutes. The sample was centrifuged (Fisher Micro-Centrifuge model 235V) for five minutes at maximum speed and the supernatant was stored at 5^{o} C for a maximum of 48 hours, prior to spotting onto nitrocellulose slides.

On the day extracts were arrayed, two-fold dilutions (1/2 to 1/128) were prepared and 70 uL of undiluted sample and each sample dilution was transferred to mapped wells of a 384-well V-bottom plate made of polypropylene (Whatman Inc., Clifton, NJ). Approximately 50 nl of each sample was spotted in triplicate onto nitrocellulose-coated glass slides. Undiluted and two-fold serial dilutions (1/2 to 1/128) of a mouse IgG (eBioscience, San Diego, CA) solution (2.5 ug/mL) were spotted on the same slides as a quality control and reference standard. Protein arraying was implemented with an 8-pin arrayer (VP478) according to the instructions of the manufacturer (V&P Scientific, Inc., San Diego, CA). Arrayed nitrocellulose slides were placed in a light-tight box that contained desiccant and stored at -20^{o} C for no more than five days prior to protein quantification.

One day prior to antibody labeling, the protein arrays were treated with Re-Blot^{TM} antibody stripping solution (Chemicon, Temecula, CA) for 15 min at room temperature, washed 2x 5 min in PBS, and then incubated overnight in blocking solution (1.2 g I-Block^{TM} [Tropix, Bedford,

MA] and 600 uL Tween® 20 [Sigma] dissolved in 600 mL Dulbecco's PBS) at 4^{o} C with constant rocking. Arrays were labeled with mouse anti-β-catenin primary antibody (clone 6F9); generously provided by Dr. Margaret Wheelock, Director of the Nebraska Center for Cellular Signaling, College of Dentistry, UNMC), for 2 hours at room temperature and then washed 2x 10min with TBS-T solution. Control slides were treated with mouse IgG in place of the primary antibody. All slides were treated for 30 min (in the dark, at room temperature) with IRDye^{TM} 800 CW-conjugated goat anti-mouse IgG (LI-COR Biosciences, Lincoln, NE) at a dilution of 1:2500. The slides were washed 2x 10min with TBS-T and air-dried.

Slides were scanned with the Odyssey infrared imaging system (LI-COR) at a resolution of 84 um, a sensitivity of 7.0 and a background setting of 'medium'. The images were analyzed with the Odyssey software. Integral fluorescence intensity, corrected for background, was determined for each spot and the mean of each set of triplicate spots was determined.

Quantitative Fluorescence Labeling

General procedures. Slide specimens of paraffin-embedded cores and tissue sections were deparaffinized with EZ-AR Common™ solution (BioGenex Laboratories) and treated for epitope recovery with EZ-AR1™ (BioGenex Laboratories) according to manufacturer specifications. Specimens were processed with a computer-controlled, microwave system (EZ-Retriever; BioGenex Laboratories). Slides were rinsed with tap water and then partially dried for application of labeling barriers with a PAP pen. Labeling was completed with the BioGenex autostainer optimized to obviate slide batching, which was associated with about a 20% loss of β-catenin signal. Moist slides were loaded into the autostainer programmed to soak slides for 15 min with Super Sensitive™ Wash Buffer, block for 20 min with 10% normal goat serum (Zymed Laboratories, Inc., San Francisco, CA), label for one hour with mouse monoclonal antibody (1^{o}

Ab) specific for β-catenin (clone CAT-5H10; Zymed Laboratories) and then label for one hour with goat anti-mouse IgG antibody (2^o Ab) coupled with Alexa Fluor® 568 (Molecular Probes, Inc., Eugene, OR). This two-antibody system superseded an avidin/biotin labeling system that produced higher backgrounds and lower signal-to-noise ratios. Quantitative fluorescence labeling requires saturation of epitopes by their corresponding Abs. Saturating dilutions of the 1^o Abs were determined by titration against β-catenin in LNCaP cells and slide preparations of a standard BH gland. The optimum dilution for saturation labeling with primary Ab was 1/100 for tissue sections and cell lines. The optimum dilution for labeling with the 2^o Ab was also 1/100. Negative controls were treated with a non-immune mouse IgG isotype (eBioscience, San Diego, CA) adjusted to an immunoglobulin concentration the same as that of the diluted 1^o Ab. The autostainer program ended, by rinsing the slides with Super Sensitive™ Wash Buffer. All labeling procedures were completed at room temperature. Labeled specimens were mounted with ProLong® Gold antifade reagent (Molecular Probes, Inc.), sealed with clear lacquer, and stored at -20^o C. Evaluation of ProLong® Gold antifade reagent demonstrated no detectable photobleaching with repeated exposures of individual specimens to excitation energy, or loss of fluorescence signal during storage at -20^o C for up to two months. Other antifade reagents including N-propylgallate (Sigma-Aldrich Co., St. Louis, MO) (26) and *SlowFade*® Antifade (Molecular Probes, Inc.) were associated with loss of fluorescence signal due to photobleaching or storage of the slide specimens.

Archived core biopsies of forty-two cases and matched controls. Each of six sets of seven pairs of matched core specimens (a total of 42 pairs of cases and matched controls) were incorporated into two separate analyses for β-catenin labeling. The first analysis included duplicate slides of seven cancer cases and seven matched controls, six slide preparations of the tissue standard and six slide preparations of the LNCaP cell standard (a total of 40 slides). Pairs

of standard slides were distributed evenly among pairs of the core specimens in the autostainer slide racks. One slide of each pair of cores and standards served for labeling β-catenin and the other served as an isotype control. The second analysis, completed on a different day, was a replica of the first analysis, providing quantification of β-catenin in duplicate slide specimens of each case and matched control. Repeat analyses of the remaining five sets of paired cases and controls were completed according to the same design, with one change. Two cases and two controls from the set of seven pairs of specimens previously analyzed were included as another level of quality control. In the event that fluorescence analysis of the 'carry-over' specimens or the standards changed by 15% or more, it would be necessary to repeat the assay and correct potential problems. Repeat assays were not required in the present study.

Fluorescence Imaging and Image Capture

Instrumentation. Quantifiable images of fluorescent, slide specimens were generated with a fully automated Leica DMRXA2 microscope (North Central Instruments, Plymouth, MN) equipped with a Marzhauser eight-slide scanning stage (North Central Instruments) and a 150-watt mercury/xenon (Hg/Xe) excitation lamp (Model E7536; Hamamatsu Corp., Bridgewater, NJ) that offers stable illumination, essential for fluorescence quantification, and a long lifetime of *ca.* 2000 hours. Relative to background illumination and fluorescence emission of the InSpeck™ standard microspheres (6 microns; excitation 505 nm/emission 515 nm) (Molecular Probes, Inc., Eugene, OR), the Hg/Xe lamp was adjusted to produce even, field illumination. The microscope filter turret included a Chroma DAPI 31000 set, a Chroma FITC 41001 set, and a Chroma Texas Red 41004 set for quantification of Hoechst, Alexa Fluor® 488, and Alexa Fluor® 568 signal, respectively. Typically, a 10x HC PLAN APO (0.40 aperture) or 20x HC PLAN APO (0.70 aperture) objective was used to capture signal from tissue sections and single cells, respectively. An E Plan 4x (0.10 aperture) objective was used to capture total signal from

tissue sections matched to those analyzed by RPPA. All microscope objectives were calibrated with an 'Objective Micrometer' (Fisher Scientific) with 0.01 mm divisions, and micrometer images were captured and stored as 12-bit 'tif' files for periodic calibration checks.

Twelve-bit grayscale images were captured for quantification of fluorescence signal, with a digital b/w CCD camera (Model ORCA-C4742-95-12ER; Hamamatsu Corp.) with high resolution (1344 x 1024 pixels), high quantum efficiency (above 50% from 400 to 700 nm), and a dynamic range of 2250:1. The camera was mounted *via* a 1.0x-coupling lens that produced an 11x magnification. Live images were visualized with a 22-inch (16 x 10 aspect ratio), high-resolution (3900 x 2400 pixels) display monitor (Eye Smart ES-100 LCD; Meyer Instruments, Inc., Houston, TX). Twelve-bit digitized images were stored as 'tif' files.

All components of the automated imaging analysis system were controlled and fully programmable with Image-Pro® Plus software (Meyer Instruments, Inc.) that included the Scope Pro® and Advanced Fluorescence Acquisition® modules. Principal features of the computer workstation (Precision 340 Workstation P4 by Dell Corporation; Meyer Instruments, Inc.) included an Intel® Pentium® 4 processor with 2.0GHz front side bussing, 1GB RAM memory (PC800), 533 MHz system bus, 32 MG, VGA graphics card, dual 80 GB hard drives (IDE 7200 rpm), USB and PCI ports and the Windows 2000 operating system.

Image capture. Each image capture session was initiated with a protocol to confirm consistent, system performance across analytical runs. Individual slides were prepared with each of three suspensions of InSpeck™ fluorescent microspheres that produced relative emissions of 1%, 10%, and 100%. Fluorescence images were generated with the 20x objective and Chroma FITC filter set and captured as 12-bit gray scale files, at exposures of 250, 25, and 3 milliseconds, respectively, with the b/w CCD camera. The 12-bit gray scale consists of 4096 divisions or units (gsu). Images of 200-400 individual microspheres per slide were segmented at a threshold of 900 gsu and measurements including mean pixel intensity (MPI) of each

microsphere were transferred to an Excel sheet for analysis. Mean and standard deviation of the MPIs for spheres from each suspension were computed and compared to the values of previous image capture sessions.

Digital images of labeled tissue specimens and the corresponding isotype controls were captured no more than three days after the slides were processed with the BioGenex autostainer. Positively labeled specimens of cases and controls, cell standards, or tissue standards were reviewed for fluorescence intensity; one specimen exhibiting the brightest fluorescence was selected from each group. Camera exposure time was set such that the brightest pixels in the selected specimen yielded values of *ca.* 3200 gsu, *i.e.*, 80% of maximum on the 12-bit gray scale. All frames were captured at this exposure setting, typically 100-200 milliseconds at a gain of '0' for both single cells and tissue sections captured with the 10x objective. Camera frames were selected to maximize the number of events (single cells, acini or epithelial strips) captured. In addition, frames of cancerous cores were selected to provide a balanced representation of cancerous acini/epithelium (CA) and non-cancerous acini (NAA). Categories of acini/epithelium included CA, NAA, and non-cancerous acini (NA) of control cores and the tissue standard. Typically, 30-300 acini were captured in each category and 200-400 single cells were captured from each slide preparation of the LNCaP cells. Images were stored as 12-bit gray scale files.

Labeled specimens matched to those specimens analyzed by RPPA were treated differently. Contiguous, non-overlapping images of antibody-labeled specimens and isotype controls were captured with the 4x objective to encompass the entire tissue section.

Quantification of Fluorescence Signal in Captured Images

Captured image files were loaded into the Image-Pro® Plus environment and relevant events, *i.e.*, individual LNCaP cells and the acini/epithelium of cores and tissue sections were segmented for quantification of fluorescence signal and event dimensions, with the 'count/size' function of

Image-Pro® Plus. Precise segmentation of prostate epithelium was achieved by setting a lower threshold just above background emission, typically *ca*. 230-250 gsu, and an upper threshold at the upper limit of the 12-bit gray scale, *i.e.*, 4095 gsu. In addition, an image filter set for a feret (event caliper measurement) of 10-15 microns excluded from measurement stromal elements, primarily endothelium, that labeled with the fluorescent reagents. Image-Pro® Plus generated a high-content array of measurements that included event number, area, feret, mean pixel intensity (MPI), sum of pixel intensities, and maximum and minimum pixel intensity. The algorithm for event MPI excluded the background pixels of each segmented event. Fluorescence signal and spatial features of the LNCaP cells were quantified in the same way, except that a filter was set for an area of 3 μ^2 and excluded fluorescent fragments from measurement.

Quantification of the β-catenin signal in slide specimens matched to those analyzed by RPPA did not entail precise segmentation of acini, but instead segmentation of the entire piece of tissue in the camera frame. In this way, the sum of pixel intensities corrected for background was determined for the entire tissue section for comparison to total β-catenin content of the paired tissue section as determined by RPPA. More than 99% of the β-catenin signal was present in the epithelium of these specimens.

Data Analysis

β-Catenin expression under different analytical conditions. Concordance of β-catenin expression in methacarn-fixed tissues analyzed by RPPA and QFIA, and in methacarn-fixed and formaldehyde-fixed tissues analyzed by QFIA was evaluated by linear regression analysis. The correlation coefficient (R value) was calculated as an indicator of the strength of the linear relationship.

β-Catenin expression in the archived core biopsies. MPIs of labeled acini/epithelium and single cells were corrected for non-specific immunoglobulin labeling and background emission by subtracting the average MPI (AMPI) of acini/epithelium in the corresponding isotype control (typically 250-300 gsu). To determine whether the AMPI in each data set was representative, we plotted the AMPI of increasing numbers of events. AMPI was considered representative if the plot attained a steady 'zero' slope, typically beginning at 30 acini or 100 cells. Arithmetic average, standard deviation, and standard error were computed for each type of event (single cell, acinus or epithelial strip). Differences between the AMPIs of event categories (NA, NAA, CA; see page 16) for each matched pair were evaluated by a paired T-test with PlotIT® software (Scientific Programming Enterprises, Haslett, MI).

Receiver operating characteristic (ROC) curves were generated for case-associated AMPIs of CA or NAA in relation to control-associated AMPIs of NA, plotted as a function of increasing threshold AMPI (27). Fractional area-under-curve (AUC) along with 95% confidence intervals was determined for each plot. An AUC ≥ 0.67 indicated significant discrimination of cases (NAA or CA) and controls (NA). Sensitivities and specificities and their 95% confidence intervals were determined at selected threshold values. Bootstrapping was used to estimate confidence intervals for the AUC and for the sensitivity and specificity at selected threshold values, taking into consideration the repeated measures design of the tests (two slides per person). Two-hundred bootstrapped samples were used to estimate the confidence intervals, resampling at the person level within a group (NA, NAA, and CA) to preserve the correlation structure from two slides within a person (27).

RESULTS

Reproducible Quantification of β-Catenin in Slide Preparations of Formaldehyde-Fixed LNCaP Cells and Prostate Tissue Sections, by Quantitative Fluorescence Imaging Analysis

Precision and reproducibility, essential prerequisites for quantitative analysis, were assured with quality control procedures, including a protocol for routine evaluation of the performance of the imaging system. This protocol, based on fluorescence emission of standard fluorescent microspheres, was completed at the start of each image capture session. AMPI was determined for three sets of microspheres with relative intensities of 1%, 10%, and 100%. A variation of greater than 5% from any previous session, for any set, would indicate the need for optical alignment, adjustment of field illumination or replacement of the Hg/Xe lamp. Fluorescence of the standard microspheres varied less than 5% among the image capture sessions required for this study as exemplified by six of these system evaluation routines **(Figure 1)**.

Analytical reproducibility was established by incorporation of both single-cell and tissue standards into each analytical run. Triplicate slides were labeled for β-catenin and negative controls were included for background correction. Reproducibility is exemplified by the data from six analytical runs **(Figure 1)**. Within each run, AMPIs of individual slides differed from the means of triplicate slides by an average of 5% for LNCaP cells and 6% for acini, and across experiments, the means of triplicate slides differed from the mean of the AMPIs of all slides by an average of 7% for LNCaP cells and 10% for acini.

Validation of Quantitative Fluorescence Imaging Analysis, Part I: Reliable Quantification of β-Catenin in Slide Specimens of Methacarn-Fixed, Paraffin-Embedded Prostate Tissue

The first step in the process of validating QFIA of β-catenin in archived specimens was to examine the reliability of quantitative determinations in methacarn-fixed tissues. RPPA was selected for this study because this well-established methodology is routinely applied to quantification of proteins in slide specimens and exhibits high precision, reproducibility and sensitivity over a wide range of protein content (11). Since our goal was to analyze β-catenin expression in paraffin-embedded, archived specimens, we elected to implement this stage of validation, with methacarn-fixed, paraffin-embedded prostate specimens. Proteins are extracted efficiently from slide specimens of tissues fixed with methacarn (28). Finally, because by far most of the β-catenin signal is expressed in prostate epithelial cells, total protein in each tissue section was analyzed, *i.e.*, specimens were not microdissected for RPPA nor were the acini of captured images segmented for QFIA. The study design required parallel analyses by RPPA and QFIA, of adjacent serial sections mounted on glass slides, maximizing agreement of protein expression in the specimens to be analyzed.

Visual inspection of the scans for one set of protein arrays corresponding to two BH glands and three cancerous glands revealed consistent size and intensity of the triplicate spots (**Figure 2, panel A**). The integrated fluorescence intensity of each spot was background corrected and the mean for each triplicate was calculated. The means for each dilution of the standard and each extract were plotted against the dilution factor (**Figure 2, panel B**). For all specimens, integrated fluorescence intensity was linear over a wide range of protein concentrations, permitting reliable comparisons across samples and analyses.

Concordance of RPPA and QFIA of β-catenin was evaluated with methacarn-fixed specimens from two BH glands and eight cancerous glands, previously screened by QFIA and selected to provide a wide range of β-catenin expression. The set of tissues represented in

Figure 2, panel A was analyzed in two separate studies and a second set of five cancerous glands was analyzed in another study. Data from the three studies were pooled and integrated fluorescence intensity of each tissue section, determined by QFIA was plotted as a function of integrated intensity of the matched tissue section, determined by RPPA (**Figure 3, Panel A**). The data exhibited a strong linear relationship with a correlation coefficient of 0.97, indicating that the QFIA measurements were directly proportional to the protein content of the tissue specimens.

Validation of QFIA, Part II: Concordance of β-Catenin Expression in Formaldehyde-Fixed and Methacarn-Fixed Prostate Tissue Specimens, determined by Quantitative Fluorescence Imaging Analysis

Concordance of QFIA measurements with β-catenin content determined by RPPA was evaluated with methacarn-fixed slide specimens, because methacarn is an accepted fixative for both RNA and protein analyses and is compatible with efficient protein extraction (28). In contrast, formaldehyde, a cross-linking fixative, is incompatible with protein extraction (29). Since we were interested in applying QFIA to archived tissues that were fixed in formaldehyde-based fixative, it was necessary to demonstrate concordance of β-catenin expression in methacarn-fixed and formaldehyde-fixed tissues, as determined by QFIA. This was implemented with a tissue microarray (TMA) that incorporated both methacarn-fixed and formaldehyde-fixed specimens from the same surgically removed cancerous and non-cancerous prostate glands. The TMA permitted a direct comparison of expression in the NA of two BH glands, the NAA of five cancer-bearing glands, and the CA of one cancer-bearing gland. To increase the stringency of this test, we included analyses of the NAA of three additional PC glands selected for relatively high expression of β-catenin. These glands were not represented in the TMA. The data from two separate analyses of the TMA specimens and a single analysis of

the additional PC glands were examined by linear regression analysis. The AMPIs of acini/epithelium in tissues fixed with formaldehyde were plotted as a function of the AMPIs of acini/epithelium in tissues fixed with methacarn, and the correlation coefficient was calculated (**Figure 3, Panel B**). AMPIs of formaldehyde-fixed and methacarn-fixed tissues exhibited a strong, linear relationship with a correlation coefficient of 0.86. We concluded that QFIA measurements of β-catenin expression were directly proportional to the quantity of β-catenin in the tissue sections and, consequently, QFIA may be applied to quantification of the protein in archived prostate specimens fixed in a formaldehyde-based fixative.

β-Catenin Expression in Archived Core Biopsies as a Potential Field Marker for Prostate Cancer

The preceding work established the foundation for application of tissue-based QFIA to the problem of quantifying β-catenin in archived prostate biopsies fixed in formalin, for an evaluation of protein expression in the NAA of cancer-bearing glands as a potential field marker for the presence of prostate cancer. Expression of β-catenin was analyzed in a cross-sectional case-control study that included 42 cancer cases and 42 controls matched on the basis of age (±5 years) and year of biopsy (±3 years). Clinical and demographic data are summarized in **Table 1**. AMPIs of the NA of non-cancer controls, and both the NAA and CA of the cancer cases were determined with gray scale images (**Figure 4, Panel A**) captured from duplicate slide specimens analyzed separately in different analytical runs. Occasional, prostatic intra-epithelial neoplasia (PIN) lesions were analyzed separately. An average of two PIN lesions was seen in specimens from each of six cancer cases. AMPIs corrected for background fluorescence are presented in **Table 2**. Consistent with reports (23) that β-catenin is principally a cell adhesion molecule in the prostate epithelium, fluorescence signal was seen in association with the cell membrane and not in the nucleus. Expression in CA compared to NA was significantly ($P<0.02$) reduced in 37 of

the 42 matched pairs of cases and controls. The grand mean ± the standard error of the AMPIs of CA for all cases was 458 ± 21, compared with 674 ± 14 for NA of all controls. In relation to altered β-catenin expression in NAA as a possible field marker for prostate cancer, it was of interest to find that expression in NAA compared to NA was significantly reduced ($P < 0.02$) in 31 of the 42 matched pairs. The grand mean ± the standard error of the AMPIs of NAA for the 31 cases was 538 ± 16 compared with 684 ± 16 for NA of the matched controls.

These observations prompted an analysis of the separation of the three groups of prostatic acini/epithelium based on the AMPI of β-catenin signaling in the acini/epithelium for each case and control. Consequently, AMPIs for the NA of the controls and the NAA and CA of the cases were pooled as three separate groups and the fraction of each group at or below selected AMPIs was plotted against AMPI (**Figure 4, Panel B**). The analysis revealed that QFIA of β-catenin produced a distinct separation of the three groups of prostatic acini. The marked separation of CA from NA supports the application of QFIA to the study of β-catenin expression in archived prostate biopsies. The separation of NAA from NA was of particular interest in relation to detecting a potential field marker for prostate cancer and suggested the possibility of detecting a significant proportion of cancer cases, with acceptible specificity. For example, none of the controls were represented at a threshold AMPI of 500 gray scale units (gsu), compared to 30% of cases.

These findings justified a more rigorous analysis with receiver operating characteristic plots of the same data sets, *i.e.*, AMPIs of NA, NAA, and CA. First we examined the classification of cases and controls based on β-catenin expression in CA and NA, respectively (**Figure 4, Panel C**). At a cut point of 500 gsu, QFIA classified 70% (95% C.I. 56%, 84%) of cancer cases as positive, while classifying only 7% (95% C.I. 1%, 13%) of controls as positive, again supporting the utility of QFIA for analysis of β-catenin expression in archived specimens. Next, we

examined classifications based on β-catenin expression in NAA and NA (**Figure 4, Panel D**). At a cut point of 500 gsu, QFIA classified 24% (95% C.I. 12%, 36%) of cancer cases as positive, with a specificity of 93% (95% C.I. 87%, 99%). Moving the cut point to 550, we find that QFIA correctly classified 42% (95% C.I. 26%, 57%) of the cases, with a specificity of 88% (95% C.I. 80%, 96%). The results indicate that β-catenin expression in NAA as determined by QFIA is potentially a useful marker for clinical diagnosis of PC in biopsies that may not include existing cancerous lesions, and support the application of QFIA to archived tissues for identification of potential markers of disease.

DISCUSSION

The human proteome consists of more than 300,000 different protein species generated *via* alternative gene splicing and post-translational modification, from an estimated 30,000 genes (2). Proteins generate useful, chemical energy and transform this energy into those functions that comprise the living organism; as a consequence, protein profiling is viewed as essential to defining disease in molecular terms. It is expected that qualitative and quantitative changes of specific proteins produce and specify disease states, offering the opportunity for prevention and early diagnosis of disease as well as individualized therapeutic intervention. This report introduces a new method, tissue-based QFIA, for quantitative analysis of proteins and secondary modifications of proteins in slide-mounted tissue sections, and demonstrates its complementation to RPPA (2, 6, 11-13) and AQUA (4, 5, 14); published methods for quantitative profiling of proteins and their secondary modifications in small, tissue samples. A key element of the present study was discriminate analysis of β-catenin expression in cancerous acini/epithelium (CA) and non-cancerous acini (NAA) in biopsies from prostate cancer (PC) patients, and non-cancerous acini (NA) in biopsies from individuals without clinical cancer. Quality controlled imaging analysis procedures, the automated imaging system, and powerful image analysis features of the Image-Pro® Plus software permitted rapid, precise analysis of all classes of acini/epithelium.

The same analysis by RPPA or AQUA is expected to encounter significant challenges. RPPA requires laser microdissection of 2,500 to 15,000 cells for preparation of extracts with sufficient protein content for analysis; this number of epithelial cells free of contaminating cells or stroma is readily achieved with NA or NAA. The same numbers of uncontaminated, cancerous epithelial cells may require many more dissections, especially in some higher-grade tumors exhibiting loss of tissue architecture and consisting of strips of relatively few, cancerous epithelial cells. Further, analysis of physically separated cells such as individual stromal cells by

RPPA may be impractical, though readily achievable by tissue-based QFIA. Finally, RPPA is not applicable to archived tissues fixed in formaldehyde-based fixative. RPPA has its strength in the analysis of multiple, extractable proteins in readily dissected tissue elements.

The application of AQUA to the present study would entail a different set of difficulties in comparison to RPPA. AQUA is a fully automated method that makes use of fluorescence labeling, *e.g.*, with an anti-cytokeratin antibody mixture, to delineate tissue compartments, *e.g.*, prostate epithelium, and to assign specific proteins to these compartments. Fluorescence discrimination of the CA and NAA compartments in the same biopsies may be a significant problem. For example, α-methylacyl-CoA racemase (AMACR) is expressed in prostate cancer and high-grade PIN and is an accepted PC biomarker (14). The use of this marker to determine the distribution and quantity of a protein of interest in the CA and NAA compartments would require a triple labeling protocol to identify epithelium (first label) with and without AMACR protein (second label), *i.e.*, CA and NAA, respectively, and to quantify the protein of interest (third label) within the compartment(s). Quantification becomes more difficult with increasing numbers of antibodies that may be associated with stearic hindrance to epitope binding. Discrimination between PIN and CA by AQUA is not likely, but is readily achieved with tissue-based QFIA. A major strength of AQUA is high throughput analysis of markers readily assignable to compartments delineated by fluorescence labeling.

Successful application of tissue-based QFIA to β-catenin in archived prostate biopsies fixed in formaldehyde-based fixative was in part the result of rigorous, quality-controlled procedures. Accordingly, core biopsies were selected for study, in part, because they are rapidly transferred to fixative, minimizing tissue degradation; cases and controls were matched for both age and year of biopsy; tissues were labeled with Ab reagents at epitope-saturating concentrations; fluorescence excitation was achieved with a stable Hg/Xe lamp adapted to the Leica microscope; programming of the robotic autostainer was tuned to obviate unscheduled slide batching

associated with reduced fluorescence signal; performance of the imaging system was evaluated with standard fluorescent beads, before each analytical run; single cell and tissue standards were included in each analytical run; and conditions for reproducibility within and across analyses and from technologist-to-technologist were established before initiating the biomarker studies. The suitability of β-catenin as a potential field marker also contributed to the success of our field marker study. Consistent expression of this protein among individual acini or epithelial strips in the three classes of prostate epithelium facilitated significant discrimination β-catenin measurements differing by 10-15%.

Dysregulation of the cadherin/catenin cell adhesion complex in PC provided the rationale for evaluating β-catenin as a potential, field marker for PC. Wehbi *et al.* (24) analyzed expression of cadherin proteins by immunohistochemistry (IHC), in 38 PC, 14 prostatitis, and 19 BPH specimens. Mean percent area positive for labeling with an anti-pan-cadherin antibody was 15.2%, 21.5%, and 2.6% for BPH, prostatitis, and PC, respectively. Ninety-five percent of the PC values fell below a threshold that excluded all BPH and prostatitis specimens combined; suggesting that altered expression of proteins in the cadherin/catenin complex may identify PC field disease/effect. A study by Kallakury *et al.* (21) supports this conclusion. The investigators applied IHC to an analysis of selected proteins of the catenin/cadherin complex in PC, comparing expression in the cancerous epithelium to expression in the non-cancerous epithelium of cancer-bearing prostate glands. E-cadherin, α-catenin, and p120 CTN were significantly reduced in 25%, 17%, and 45%, respectively, of 118 cancer-bearing glands. This same study, however, found significant reduction of β-catenin in only 4% of the PC cases, implying that this protein may not be a useful marker of PC. A similar study by Bismar *et al.* (23) also found reduction of β-catenin in only 4% of PC cases (N=89).

These reports seemingly contradict results obtained in the present study, which demonstrated significant reduction of β-catenin expression in the CA of 37 of the 42 PC cases. The apparent

contradiction is resolved by acceptance of the validity of the data of all three studies and appreciation of substantial differences in experimental design. Kallakury *et al.* (21) and Bismar *et al*. (23) evaluated expression in the CA in relation to that in the NAA of cancer-bearing glands, whereas in the present study, we evaluated expression in the CA of biopsies from PC cases in relation to both the NAA of the same biopsies and the NA of biopsies from matched controls without clinical disease. In addition, the present study was based on continuous quantitative measurements whereas the studies of Kallakury *et al.* (21) and Bismar *et al*. (23) were based on IHC classifications. The consequence of the latter difference is emphasized by our observation that expression of β-catenin in CA in relation to NAA of cancer-bearing glands was significantly reduced in 71% of the 42 PC cases in contrast to 4% in both IHC studies. It is interesting to note that in our study, expression in the CA of 4 of the 42 cases was reduced relative to the NAA by more than 50%; a frequency of down regulation similar to that achieved in the IHC studies.

Successful application of tissue-based QFIA to β-catenin in prostate tissues is attributed, in part, to key features of the protein. First, β-catenin exhibits bright, specific labeling in archived tissues fixed with formaldehyde-based fixative. This feature opened the tissue archives to tissue-based QFIA of the protein as a potential field marker for PC. Second, β-catenin signal was consistent among individual acini or epithelial strips within each class (CA, NAA, NA) of prostate epithelium evaluated. This feature precluded significant analytical variance that can mask biologically significant differences between the classes of epithelium. In agreement with published studies (23), we found β-catenin labeling, in all three classes, to be restricted to the cytoplasm in the vicinity of the cell membrane, and did not detect nuclear signal. On this basis, Bismar *et al.* (23) distinguish PC and colorectal cancer in which the protein is translocated to the nucleus and is detectable by IHC. The investigators suggest the presence of different β-catenin signaling pathways in prostate and colorectal carcinogenesis.

The major finding from the application of tissue-based QFIA to β-catenin in archived prostate specimens was the generally reduced expression of β-catenin in NAA relative to NA. This difference was sufficient to produce thresholds of fluorescence intensity that detected 24% and 42% of cancer cases, with specificities of 93% and 88%, respectively. The observed sensitivities and specificities do not qualify the marker as an independent indicator of PC field disease/effect, but identify altered expression of the protein as a potentially significant contributor to a small panel of field markers, and support the feasibility of such a panel. Ultimately, the diagnostic value of this marker can be determined best by studies of tissues collected prospectively and processed by rigorously standardized procedures that may further reduce measurement variance and improve the sensitivity of the QFIA method. Such procedures are integral to the 'Molecular Profiling Initiative' of the Cancer Genome Anatomy Project of the National Cancer Institute (www.cgap.nci.nih.gov) and the 'Biospecimen Banking Program' of the Department of Defense, Center for Prostate Disease Research (www.cpdr.org).

In conclusion, we have developed tissue-based QFIA, a new method for quantification of proteins and secondary modifications of proteins in precisely selected elements of slide-mounted specimens, in the context of observable histological features. We have validated by RPPA the application of QFIA to β-catenin in archived prostate tissue fixed in formaldehyde-based fixative. Reduced expression of β-catenin in NAA relative to the NA of matched controls is a potential field marker for PC, in biopsies that miss existing cancer nodules. The observed sensitivities and specificities qualify the marker as a potentially significant contributor to a small panel of field markers, and support the feasibility of applying QFIA to the development of such a panel.

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Table 1: Summary of Clinical Data for Prostate Cancer Cases and Controls

	Non-Cancer Controls		Cancer Cases
	No Noted Pathology	Glandular Hyperplasia	
Number of cases/controls	23	19	42
Mean age at biopsy (years)	66	67	67
Age range at biopsy (years)	57- 78	58 - 77	55 - 77
Year of biopsy (range)	1992 - 1998	1992 - 1996	1991 - 1997
PSA (ng/ml) at time of biopsy (range)	0.8 - 44.9	0.7 - 13.7	1.3 - 73.4
Gleason Score (range)	—	—	2 - 9
2-4 (number)	—	—	5
5-7 (number)	—	—	31
8-10 (number)	—	—	6

Table 2: Quantitative Fluorescence Imaging Analysis of β-Catenin in the Acini/Epithelium of Archived Biopsies from Prostate Cancer Cases and Controls

Controls (Acini)			**Prostate Cancer Cases**				
				Non-Cancerous Acini		Cancerous Acini/Epithelium	
Patient Code	#Average Corrected MPI	STDEV	Patient Code	Average Corrected MPI †(p value)	STDEV	Average Corrected MPI ‡(PIN)	STDEV
NPC1	783	183	Δ PC1	689 (p=0.02)	120	655	85
NPC2	733	125	Δ PC2	654 (p<0.01)	146	579	131
NPC3	786	123	Δ PC3	639 (p<0.01)	159	505	122
NPC4	627	111	Δ PC4	569 (p=0.009)	59	440	90
NPC5	826	121	Δ PC5	616 (p<0.01)	116	412	79
NPC6	746	158	Δ PC6	731 (p=0.01)	130	807(665)	142
NPC7	669	121	Δ PC7	603 (p<0.01)	76	436	94
NPC8	748	111	Δ PC8	584 (p<0.01)	98	577(611)	119
NPC9	670	123	Δ PC9	603 (p<0.01)	109	588	119
NPC10	768	110	Δ PC10	510 (p<0.01)	112	414	86
NPC11	742	121	Δ PC11	596 (p<0.01)	96	188	38
NPC12	720	131	Δ PC12	586 (p<0.01)	103	558	120
NPC13	648	89	Δ PC13	551 (p<0.01)	103	401(550)	111
NPC14	595	97	Δ PC14	537 (p<0.01)	84	410	68
NPC15	668	110	Δ PC15	544 (p<0.01)	120	563	134
NPC16	828	138	Δ PC16	595 (p<0.01)	109	487	68
NPC17	635	104	Δ PC17	537 (p=0.006)	111	521	94
NPC18	700	113	Δ PC18	610 (p<0.01)	122	429	77
NPC19	723	140	Δ PC19	586 (p<0.01)	104	445	65
NPC20	563	100	Δ PC20	449 (p<0.01)	104	381	113
NPC21	716	152	Δ PC21	472 (p<0.01)	94	233	86
NPC22	507	84	Δ PC22	450 (p<0.01)	89	185	59
NPC23	567	127	Δ PC23	352 (p<0.01)	76	335	65
NPC24	859	155	Δ PC24	438 (p<0.01)	114	221	49
NPC25	777	127	Δ PC25	491 (p<0.01)	89	354(427)	90
NPC26	549	81	Δ PC26	360 (p=0.001)	62	338	65
NPC27	581	115	Δ PC27	427 (p<0.01)	84	356	60
NPC28	625	121	Δ PC28	489 (p<0.01)	123	456	87
NPC29	565	142	Δ PC29	459 (p<0.01)	88	369	71
NPC30	643	100	Δ PC30	475 (p<0.01)	107	445	112
NPC31	624	153	Δ PC31	474 (p<0.01)	99	406	92
NPC32	579	111	Ψ PC32	772 (p<0.01)	168	453	132
NPC33	685	130	Ψ PC33	725 (p<0.01)	177	504	88
NPC34	577	124	Ψ PC34	690 (p=0.002)	167	518	107
NPC35	654	115	Ψ PC35	786 (p<0.01)	152	558	110
NPC36	681	113	Ψ PC36	749 (p<0.01)	120	649	130
NPC37	531	74	Ψ PC37	535 (p=0.01)	92	408(645)	87
NPC38	754	214	PC38	709 (p=0.43)	106	439	62
NPC39	629	86	PC39	622 (p=0.12)	108	615	76
NPC40	820	121	PC40	890 (p=0.99)	139	784	127
NPC41	675	118	PC41	714 (p=0.29)	163	411 (487)	77
NPC42	511	58	PC42	552(p=0.06)	114	411	103
Average	674	92		581	118	458	136

\# Average of the background corrected Mean pixel intensity (MPI) of β-Catenin labeling in individual acini

† Noncancerous acini (NAA) in cancerous glands compared to non-cancerous acini (NA) of the matched controls

‡ Average corrected MPI of β-Catenin labeling in prostate intra-epithelial neoplastic (PIN) lesions

Δ β-Catenin is significantly down regulated in NAA compared to NA

ψ β-Catenin is significantly up regulated in NAA compared to NA

LEGENDS

Figure 1. Stable Performance of the Imaging Analysis System and Reproducible Quantification of β-Catenin in Standard Cells and Tissue Sections: Basis for Quantitative Comparison and Data Pooling Across Analytical Runs

Fluorescence emission of each standard microsphere at 1% (black bars), 10% (gray), and 100% (open) relative intensity, captured at 250, 25, and 3 milliseconds, respectively, was expressed as background corrected mean pixel intensity (MPI). Each bar of the histogram represents the average MPI ± SEM of events (200-300 microspheres) on one slide. Triplicate slide specimens of standard LNCaP cells and a non-cancerous prostate gland were labeled with saturating dilutions of anti-β-catenin Ab and anti-mouse IgG-Alexa Fluor® 568 conjugate, in six separate analytical runs. Fluorescence emission of each event (cell or acinus) was expressed as background corrected mean pixel intensity (MPI). Each bar of the corresponding histograms represents the average MPI ± SEM of events (400-700 single cells or 100-200 acini) on one slide. The range of SEM for microspheres, LNCaP cells, and tissue sections was 4.6-6.7, 6.4-8.6, and 6.0-10.7 gsu, respectively.

Figure 2. Reverse-Phase Protein Array Analysis of β-Catenin in Slide Specimens of Prostate Tissue

One slide-mounted section (4 u) of a paraffin-embedded specimen from each of two benign hyperplastic glands (**BHG**; specimens 'AJ' and 'ED') and three cancer-bearing glands (**CaG**; specimens 'BM', 'CX' and 'CT') fixed in methacarn, was shaved from the glass slide and deposited into extraction buffer. Extracts as well as a mouse IgG standard solution (**IgG**; 250 ng IgG/100 uL) were diluted two-fold and both undiluted sample and sample dilutions were transferred to a micro-well plate. Samples were arrayed (40-50 nL/spot) in triplicate to

nitrocellulose slides that were treated, subsequently, with mouse anti-β-catenin antibody (**Ab**) and a secondary Ab (goat anti-mouse IgG coupled to an infrared fluorophore). For each sample, the top row corresponds to undiluted sample and dilutions of 1:4, 1:16, and 1:64 and the bottom row corresponds to dilutions of 1:2, 1:8, 1:32 and 1:128. A control slide arrayed with duplicate sets of mouse IgG standard diluted 1:2, 1:8, 1:32 and 1:128 and one set of dilutions (1:2, 1:8, 1:32 and 1:128) of each extract was treated with mouse IgG isotype control (**ISO**) and then with secondary Ab. Slides were scanned with an infrared imaging system and the background-corrected fluorescence of each spot was calculated as integrated intensity.

Figure 3. Validity of Quantitative Fluorescence Imaging Analysis (QFIA) of β-Catenin in Archived Prostate Specimens Fixed in Formaldehyde-Based Fixative

Part A. Reverse-Phase Protein Array Analysis (RPPA) and QFIA of β-catenin in slide-mounted, prostate specimens fixed in methacarn exhibit a linear correlation. β-Catenin in adjacent serial sections mounted on glass slides was analyzed in parallel by RPPA and QFIA. Specimens from one set (**Set '1'**) of two benign hyperplastic glands and three cancer-bearing glands were analyzed in two separate analytical runs (**Analyses '1' and '2'**). A second set of five cancer-bearing glands (**Set '2'**) was analyzed in a single analytical run. Background-corrected, integrated pixel intensity determined by QFIA was plotted against background-corrected, integrated intensity determined by RPPA for the fifteen pairs of tissue sections, and the correlation coefficient (R value) was determined with Plot-IT® software.

Part B. β-Catenin values determined by QFIA of formaldehyde-fixed and methacarn-fixed prostate tissues exhibit a strong linear relationship. The regression analysis includes measurements of β-catenin in formaldehyde-fixed and methacarn-fixed specimens from two benign hyperplastic (BH) glands and eight cancer-bearing (PC) glands. The non-cancerous acini (NA) of the two BH glands, the non-cancerous acini (NAA) of five PC glands and the cancerous

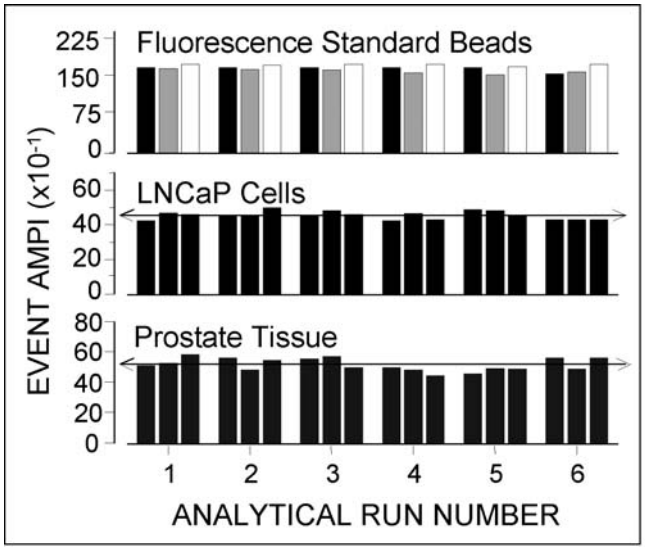
acini/epithelium (CA) of one of the five PC glands were analyzed as part of a tissue micro-array (TMA) in two separate analytical runs. The NAA of three additional PC glands were analyzed separately in a single run. The data are presented as the average of the background corrected mean pixel intensities (AMPI) of the prostatic acini/epithelium. Each data point determined with the TMA represents the mean of the two analytical runs. Each data point determined with specimens of the individual PC glands represents one analytical run.

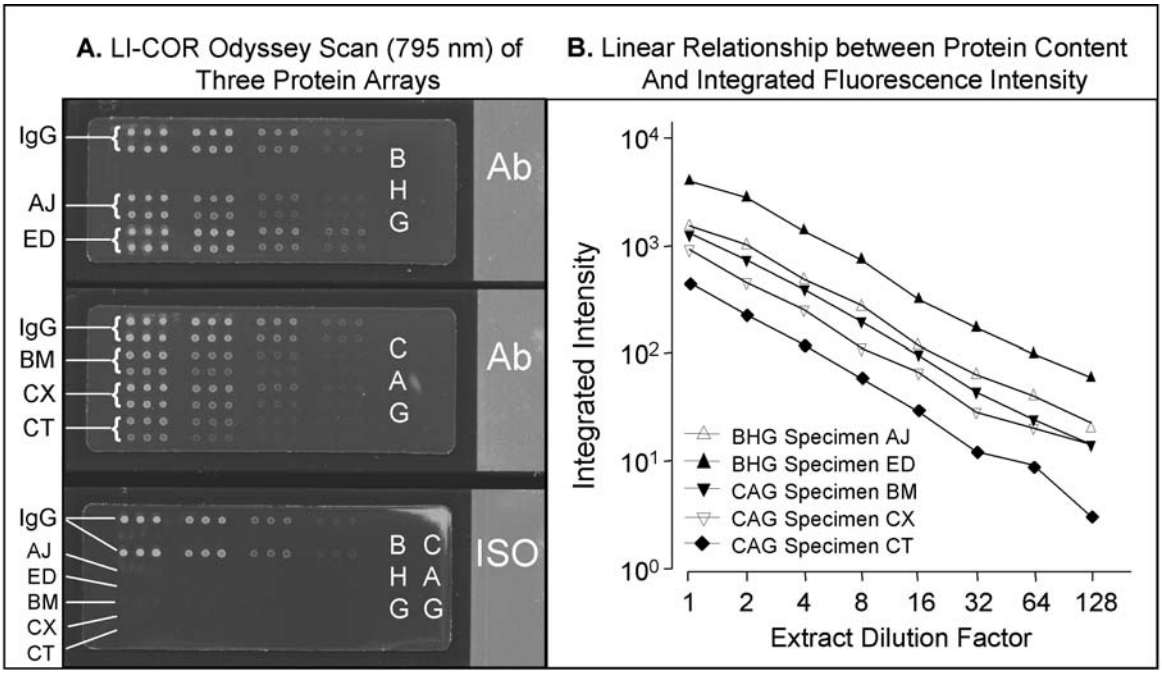
Figure 4. Discrimination of Three Classes of Prostatic Acini by Quantitative Fluorescence Imaging Analysis of β-Catenin in Prostate Biopsies

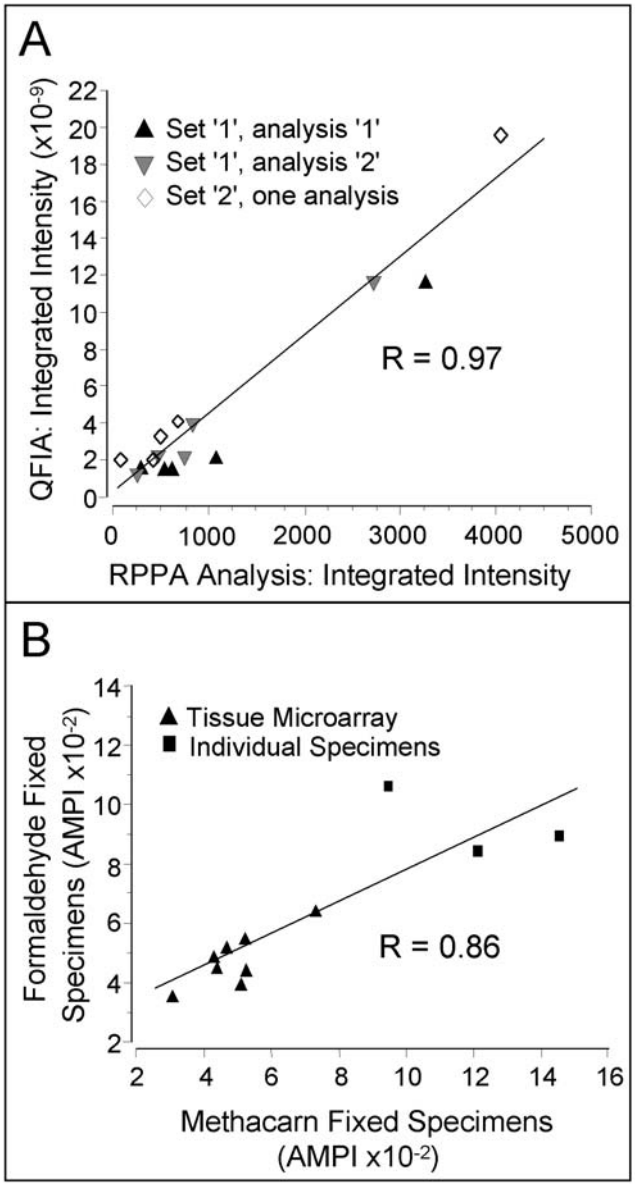
Part A. Representative gray scale images of fluorescing acini labeled for quantitative analysis of β-catenin in slide-mounted, prostate specimens. β-Catenin in archival core biopsies from a cancerous prostate gland and a matched non-cancerous control was labeled with a combination of anti-β-catenin antibody and secondary antibody-Alexa Fluor® 568 conjugate. Fluorescence images were generated with an automated Leica microscope configured with a 10x objective and a filter set for the fluorophore and captured with a Hamamatsu CCD black and white camera. Cancerous acini (**Image 1**) and non-cancerous acini (**Image 2**) of a cancer-bearing gland exhibited low-level and intermediate labeling, respectively, and non-cancerous acini (**Image 3**) of the matching control gland exhibited bright labeling.

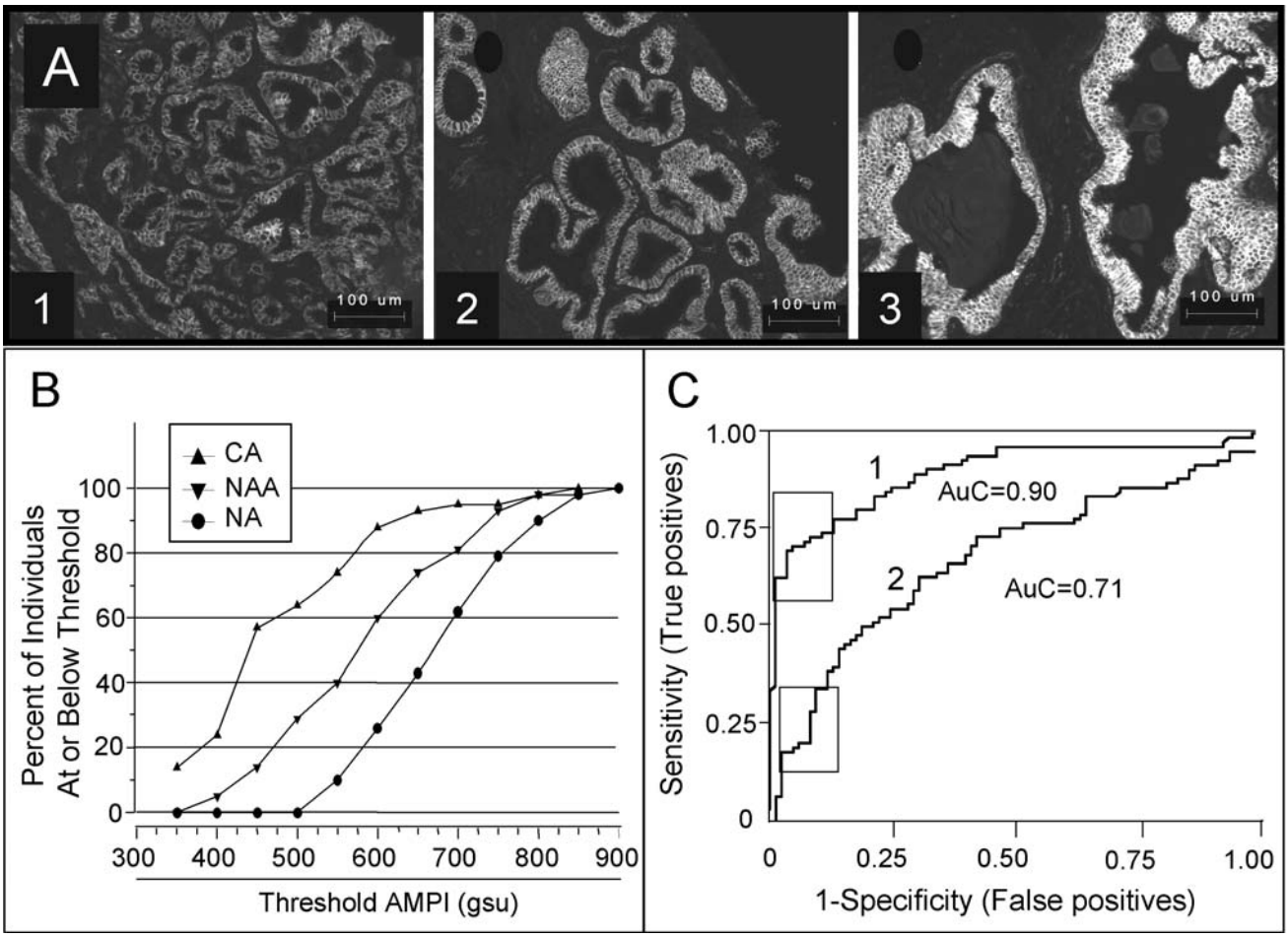
Part B. β-Catenin was analyzed in cancerous acini/epithelium (**CA**) and non-cancerous acini (**NAA**) of biopsies from 42 prostate cancer cases and in the non-cancerous acini (**NA**) of biopsies from 42 matched controls without clinical cancer. The average of the background corrected mean pixel intensity (**AMPI**) for individual classes of acini/epithelium was determined (duplicate slides) for each case and control, and the values were grouped according to class. The percentage of values in each group (NA, NAA, CA) at or below selected threshold AMPIs was plotted against threshold AMPI expressed as 12-bit gray scale units (**gsu**).

Part C. Receiver operating characteristic (ROC) analysis of β-catenin expression in cancerous acini/epithelium (**CA**) and non-cancerous acini (**NAA**) of cancer-bearing prostate glands, in relation to expression of β-catenin in the non-cancerous acini (**NA**) of matched controls. β-Catenin was analyzed in CA and NAA of biopsies from 42 prostate cancer cases and in the NA of biopsies from 42 matched controls without clinical cancer. The average of the background corrected mean pixel intensity (**AMPI**) for individual classes of acini/epithelium was determined (duplicate slides) for each case and control. The fraction of controls with NA values and the fraction of cases with CA values (**Curve '1'**) or NAA values (**Curve '2'**) at or below selected AMPI values on a 12-bit gray scale were plotted as **1-Specificity** (X-axis) and **Sensitivity** (Y-axis), respectively. AUC indicates area-under-curve and the open boxes represent the 95% confidence intervals for 1-Specificity and Sensitivity.


Fluorescence Standard Beads
225
150
75
0
LNCaP Cells
60
40
20
0
Prostate Tissue
80
60
40
20
0
EVENT AMPI (x10-1)
1
2
3
4
5
6
ANALYTICAL RUN NUMBER


A. LI-COR Odyssey Scan (795 nm) of Three Protein Arrays
B. Linear Relationship between Protein Content And Integrated Fluorescence Intensity
IgG
AJ
ED
B H G
Ab
IgG
BM
CX
CT
C A G
Ab
IgG
AJ
ED
BM
CX
CT
B C H A G G
ISO
Integrated Intensity
10^4
10^3
10^2
10^1
10^0
BHG Specimen AJ
BHG Specimen ED
CAG Specimen BM
CAG Specimen CX
CAG Specimen CT
1 2 4 8 16 32 64 128
Extract Dilution Factor


A
QFIA: Integrated Intensity (x10-9)
Set '1', analysis '1'
Set '1', analysis '2'
Set '2', one analysis
R = 0.97
RPPA Analysis: Integrated Intensity
B
Formaldehyde Fixed Specimens (AMPI x10-2)
Tissue Microarray
Individual Specimens
R = 0.86
Methacarn Fixed Specimens (AMPI x10-2)


A
1
100 um
2
100 um
3
100 um
B
CA
NAA
NA
Percent of Individuals
At or Below Threshold
0
20
40
60
80
100
300
400
500
600
700
800
900
Threshold AMPI (gsu)
C
1.00
0.75
0.50
0.25
0
Sensitivity (True positives)
1
AuC=0.90
2
AuC=0.71
0
0.25
0.50
0.75
1.00
1-Specificity (False positives)